MELANOMA-ASSOCIATED SUPPRESSION OF THE DENDRITIC CELL LINES DC2.4 AND JAWSII

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ABSTRACT

Dendritic cells function as potent regulators of both innate and adaptive immunity to tumors and the regulatory activities of these cells are tightly linked to their maturation and activation status. Despite the critical role played by dendritic cells in the induction of anti-tumor immune responses, the number of dendritic cells that can be isolated from experimental animals is limiting and often precludes in-depth analyses of these cells. To overcome this limitation, dendritic cell lines have been established and have facilitated the experimental study of dendritic cell biology. In this study we compare the dendritic cell lines DC2.4 and JAWSII as *in vitro* model systems for studying the influence of melanoma-derived factors on dendritic cell maturation and activation. Using flow cytometry and ELISA analyses, we evaluate the expression of costimulatory/MHC class II molecules and proinflammatory cytokines/chemokines by these dendritic cell lines in their resting state and following LPS stimulation in the presence or absence of B16-F1 melanoma-derived factors. Results: We demonstrate that soluble B16-F1-derived factors suppress the LPS-induced upregulation of CD40, CD80, CD86 and MHC class II on both the DC2.4 and JAWSII dendritic cell lines. Interestingly, LPS-induced secretion by DC2.4 cells of the proinflammatory cytokines/chemokines TNF-α, IP-10, MIP-1α, MIP-1β and MCP-1 is also altered by B16-F1-derived factors, whereas JAWSII cell cytokine/chemokine production is affected to a lesser extent by such factors, with only IL-1β and IP-10 production being suppressed. Conclusions/Recommendations: We conclude that melanoma-derived factors can suppress dendritic cell maturation/activation and that the DC2.4 and JAWSII dendritic cell lines are effective *in vitro* models for future studies that aim to (1) identify factors that influence both the susceptibility and the resistance of dendritic cells to tumor-mediated immunosuppression and (2) investigate the influence of tumor-altered dendritic cells on the quality of anti-tumor T cell responses.

Keywords: Melanoma, Tumor Immunity, Dendritic Cell

1. INTRODUCTION

Dendritic Cells (DC) are a population of lymphoid- and peripheral tissue-resident innate immune cells derived from lymphoid and myeloid bone marrow progenitors. DC possess a number of immunoregulatory functions and are capable of inducing either tolerance to or activation against the antigens they encounter. Importantly, the immune response elicited by DC to such antigens is dictated by the context in which these antigens are acquired and the maturation and activation status of the dendritic cell (Matzinger, 1994; Banchereau and Steinman, 1998; Janeway and Medzhitov, 2002).

Immature DC induce either immunologic ignorance or tolerance to self antigens and antigens derived from sources that do not represent "danger" to the host. On the other hand, acquisition of antigens in the presence of "danger signals" that stimulate DC maturation and activation induces DC to express costimulatory molecules and proinflammatory cytokines and chemokines that are necessary to activate and recruit T cells and other immune effectors to the source of antigen within the body for elimination from the host (Albert *et al.*, 2001; Filatenkov *et al.*, 2006; Sousa, 2006; Joffre *et al.*, 2009). Because DC maturation and activation are so critical for the induction of immunity,

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it is important to gain a better understanding of the factors that regulate these processes and the DC functions associated with them.

Immunity to cancer has long been appreciated (Broek *et al*., 1996; Shankaran *et al*., 2001) and since the emergence of the field of tumor immunology, several studies have subsequently described mechanisms of tumor immune escape to explain the outgrowth of tumors in immunocompetent hosts (Rabinovich *et al*., 2007). The majority of these studies report mechanisms of escape from tumor antigen-specific T cells, either by evasion of activated, effector T cells (through dowregulation of tumor antigen/MHC molecule expression (Restifo *et al*., 1993; Garrido *et al*., 1995) or induction of T cell death (Hahne *et al*., 1996; Dong *et al*., 2002) or by suppression of effector T cell function (Radoja *et al*., 2001; Blohm *et al*., 2002; Mortarini *et al*., 2003; Anichini *et al*., 2003; Whiteside *et al*., 2004; Koneru *et al*., 2005; Hargadon *et al*., 2006). Because of the importance of DC in regulating immune responses, it is appealing to speculate that tumors might also interfere with DC function. However, while several studies have identified an immunosuppressive role for DC-like precursors known as tumor-associated immature myeloid cells (Almand *et al*., 2001; Terabe *et al*., 2003; Kusmartsev *et al*., 2004; Huang *et al*., 2006; Ostrand-Rosenberg and Sinha, 2009), little is currently known about the influence of tumors on the function of fully differentiated DC. This lack of information is due in large part to the limiting numbers of DC *in situ* and the associated difficulties inherent in isolating these cells from tumor-bearing patients and animals for experimental analyses. Importantly, though, DC lines that can be maintained *in vitro* have recently been established to overcome these limitations and facilitate the study of DC immunobiology (Shen *et al*., 1997; MacKay and Moore, 1997; Helden *et al*., 2008). Using the C57Bl/6-derived DC2.4 cell line, we have previously reported that B16-F1 melanoma-derived factors are capable of suppressing the LPS-induced expression of costimulatory molecules and proinflammatory cytokines/chemokines by these cells and that the extent of this suppression correlates with the tumorigenicity of the melanoma variant under study (Hargadon *et al*., 2012). In this report, we compare the melanoma-induced suppression of the DC2.4 cell line with that of another commonly used C57Bl/6-derived DC line, JAWSII, in order to establish the efficacy of these lines as *in vitro* model systems for studying melanoma-associated suppression DC maturation and activation and to determine whether the quality of this immunosuppression is dependent on the DC line under study. The significance of our results and the utility of these DC lines as models for studying melanoma-associated suppression of DC function are discussed herein.

2. MATERIALS AND METHODS

2.1. Cell Lines

DC2.4 cells are an adherent C57Bl/6 bone marrow-derived DC line and were kindly provided by Dr. Kenneth Rock (Dana Farber Cancer Institute, Boston, MA). The JAWSII cell line was purchased from American Type Culture Collection (Manassas, VA) and is a GM-CSF-dependent, C57Bl/6 bone marrow-derived DC line that grows as a mixture of adherent and suspension cells. B16-F1 murine melanoma cells were a generous gift of Dr. Victor Engelhard (University of Virginia). The DC2.4 and B16-F1 cell lines were grown in RPMI-1640 medium (Thermo Scientific, Hudson, NH) supplemented with 10% fetal bovine serum (Premium Select, Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 2 g L^{-1} glucose, 2 g L^{-1} sodium bicarbonate and a mixture of 100 U/mL penicillin and 100 µg/mL streptomycin (ATCC). JAWSII cells were grown in HyClone Iscove's Modified Dulbecco's Medium (Thermo Scientific) supplemented with 10% fetal bovine serum (Premium Select, Atlanta Biologicals), 4 mM L-glutamine, HEPES, 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Sigma), a mixture of 100 U/mL penicillin and 100 µg mL^{-1} streptomycin (ATCC) and 5 ng/mL recombinant murine GM-CSF (Thermo Scientific). Cells were passaged at 80-90% confluence.

2.2. Antibodies and Reagents

The following monoclonal antibodies were purchased from eBioscience (San Diego, CA) and used in the analysis of DC2.4 and JAWSII maturation: anti-CD40 PE (1C10), anti-CD80 PE (16-10A1), anti-CD86 PE (GL1) and anti-MHC class II (I-A/I-E) PE (M5/114.15.2). LPS was purchased from Invivogen (San Diego, CA).

2.3. Generation of Tumor-Conditioned Media (TCM)

B16-F1 tumor cells (1×10^6 cells/well) were plated in 6-well tissue culture plates. Twenty four hours later, culture supernatants were collected and centrifuged at 1,000g for 10 min to remove any cellular debris. The resulting supernatant was used as TCM for experiments as indicated.

2.4. Maturation/Activation of DC Lines

DC2.4 or JAWSII cells growing in culture were harvested, resuspended in appropriate growth media ± 1

µg mL^{-1} LPS and plated at 1×10^6 cells/mL in 6-well tissue culture plates. In some experiments, DC were resuspended in TCM ± 1 µg mL^{-1} LPS. After 24 h, cells were collected by cell scraping and fixed for flow cytometry staining. At this time, cell culture supernatants were also collected by centrifugation at 1,000 g to remove any cellular debris and frozen at -20° C for subsequent ELISA analysis of cytokine and chemokine production.

2.5. Flow Cytometry Analysis

Cells were collected from *in vitro* cultures and fixed in 2% paraformaldehyde for 10 min at room temperature. Cells were then resuspended in FACS Buffer (PBS supplemented with 0.5% BSA and 0.02% NaN$_3$) and Fc blocked for 5 min at 4°C, followed by surface staining with the indicated monoclonal antibodies for 30 min at 4°C in the dark. Labeled cells were detected by flow cytometry using a FACS Canto (BD, Mountain View, CA) and were analyzed using Flowjo software (Tree Star).

2.6. ELISA

ELISA analysis for the indicated cytokines and chemokines was performed using Ready-Set-Go! ELISA kits from eBioscience (San Diego, CA) and SABiosciences ELISArray kits from Qiagen (Frederick, MD) according to the manufacturers' protocols.

2.7. Statistical Analyses

Values are expressed as the mean ±SD and differences among indicated groups were analyzed using unpaired t tests. A value of $p \leq 0.05$ was considered significant and is represented in graphs by *. ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

3. RESULTS

3.1. The Influence of B16-F1 Melanoma-Derived Factors on LPS-Induced Maturation of DC2.4 and JAWSII DC Lines

B16-F1 is a highly aggressive murine melanoma that grows progressively and metastasizes upon tumor challenge of mice (Fidler, 1975). This tumor has also been shown to induce dysfunctional anti-tumor CD8$^+$ T cell responses (Hargadon et al., 2006) that are similar to those often observed in melanoma patients (Mortarini et al., 2003; Anichini et al., 2003; Zippelius et al., 2004) and it has been suggested that this dysfunction may arise from an influence of the tumor on the functional quality of DC. Because of the inherent difficulties in isolating tumor-associated DC from B16-F1 tumor-bearing animals, though, it has thus far not been possible to thoroughly examine the nature of these cells. Therefore, we wished to employ an *in vitro* model system to directly study the effects of B16-F1-derived factors on DC maturation and activation. To this end, we stimulated DC2.4 and JAWSII DC lines with LPS in either normal growth media or B16-F1 tumor-conditioned media (TCM) for 24 h. In the absence of B16-F1-derived factors, LPS induced both DC lines to upregulate their expression of MHC class II and the costimulatory molecules CD40, CD80 and CD86 (**Fig. 1**).

Interestingly, when both the DC2.4 and JAWSII DC lines were stimulated with LPS in the presence of B16-F1 TCM, the upregulation of MHC class II, CD40, CD80 and CD86 was suppressed (**Fig. 1**). These data demonstrate that soluble factors derived from B16-F1 melanoma suppress the maturation of DC2.4 and JAWSII DC.

3.2. The Influence of B16-F1 Melanoma-Derived Factors on LPS-Induced Proinflammatory Cytokine/Chemokine Expression by DC2.4 and JAWSII DC Lines

Our previous data regarding LPS-induced maturation of the DC2.4 and JAWSII cell lines confirm findings from other studies (He et al., 2007; Rhule et al., 2008; Jiang et al., 2008) demonstrating that these cells are relatively immature in their resting state and can be induced to upregulate expression of costimulatory and MHC class II molecules. However, an in-depth characterization of the cytokines and chemokines produced by resting and LPS-stimulated DC2.4 and JAWSII cells has yet to be reported. Therefore, in order to assess the cytokine and chemokine profiles of these DC lines, cells were left untreated or were exposed to LPS for 24 h and cell culture supernatants were then collected for ELISArray analysis of a panel of cytokines (**Fig. 2A**) and chemokines (**Fig. 2B**). Following LPS stimulation, both DC2.4 and JAWSII DC upregulated their expression of the proinflammatory cytokines IL-1α, IL-6 and TNF-α. Additionally, the JAWSII cell line also upregulated expression of IL-1β after stimulation with LPS. Likewise, LPS stimulation induced both cell lines to increase their expression of a number of chemokines, including RANTES, MCP-1, MIP-1α, MIP-1β and IP-10.
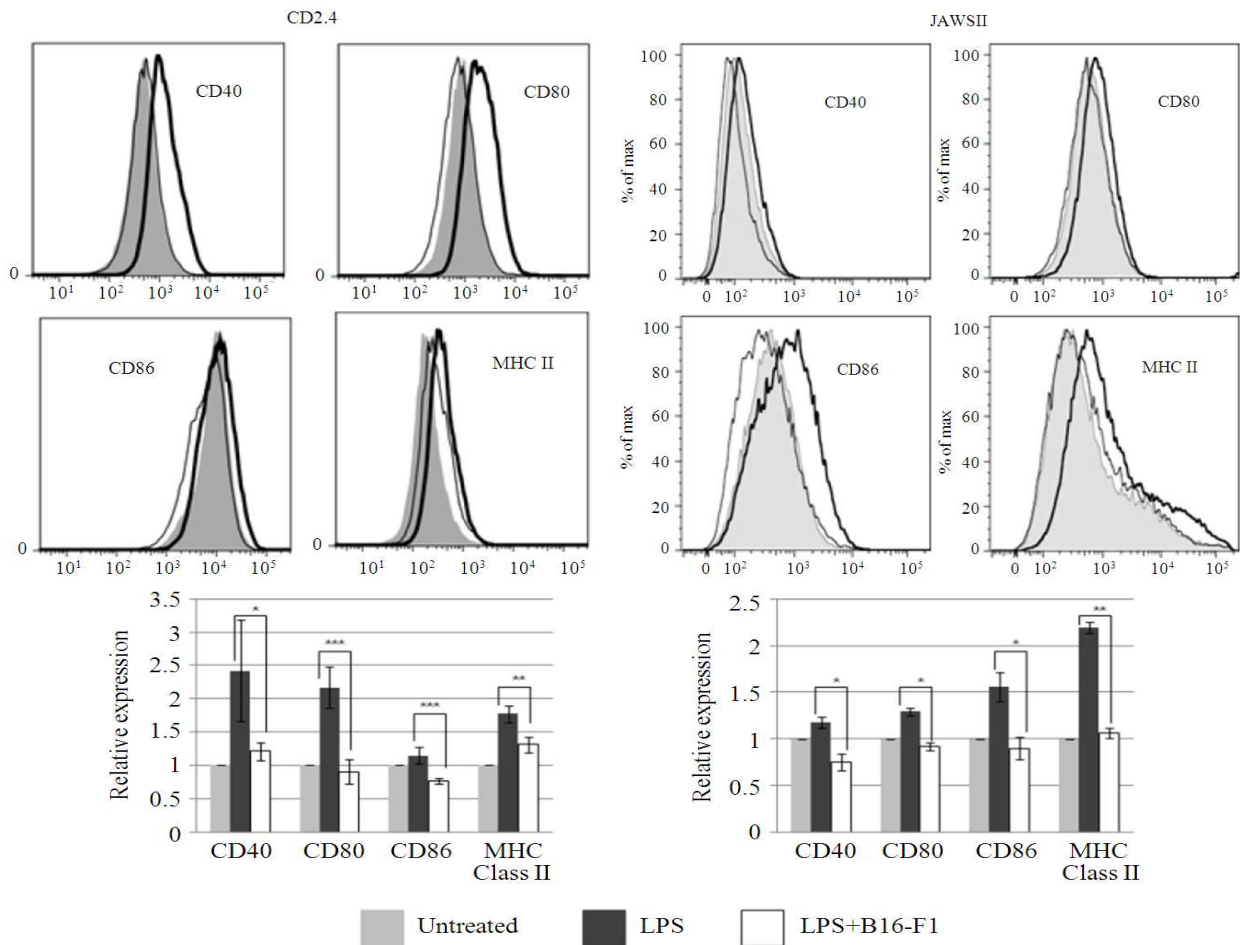
Fig. 1. B16-F1 tumor-altered maturation of DC2.4 and JAWSII DC. DC2.4 (A) and JAWSII (B) cells were cultured for 24 h in normal growth media (shaded histogram), growth media containing 1 µg mL^{-1} LPS (bold-lined histogram), or TCM derived from B16-F1 supplemented with 1 µg mL^{-} LPS (thin-lined histogram). Following culture, cells were harvested and maturation status was assessed by staining with the indicated antibodies for flow cytometric analysis. Relative expression was calculated as the fold change in the geometric mean of fluorescence as compared to untreated cells. Histogram data are representative of 4 independent experiments and the bar graph depicts the mean ± SD of these 4 independent experiments

Because B16-F1-derived factors suppressed LPS-induced costimulatory/MHC class II molecule expression by DC2.4 and JAWSII DC, we wished to determine whether tumor-derived factors might also alter cytokine and chemokine production by these cells. Therefore, we stimulated DC2.4 and JAWSII DC lines with LPS in either normal growth media or B16-F1 TCM for 24 h and performed quantitative ELISA analysis on cell culture supernatants to assess production of the cytokines/chemokines identified in the ELISArray reported in **Fig. 2**. As shown in **Fig. 3A**, DC2.4 production of several cytokines/chemokines was altered when these cells

were stimulated with LPS in the presence of B16-F1 TCM. TNF-α production was augmented when these cells were exposed to soluble factors derived from the B16-F1 melanoma. These tumor-derived factors also suppressed the LPS-induced secretion of the chemokines IP-10, MCP-1, MIP-1α and MIP-1β by DC2.4 cells. Interestingly, LPS-induced cytokine/chemokine secretion by JAWSII DC was affected to a lesser extent by B16-F1 tumor-derived factors (**Fig. 3B**). With the exception of suppressed IL-1β and IP-10 production, JAWSII cytokine and chemokine secretion patterns were not altered when these cells were stimulated with LPS in the presence of B16-F1 TCM.
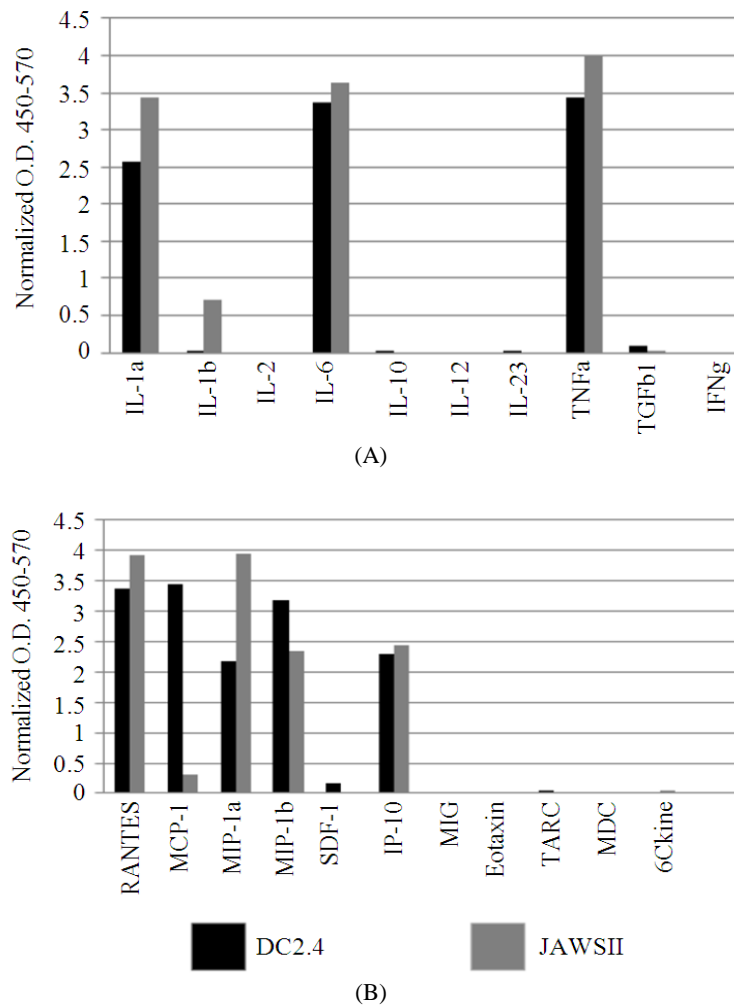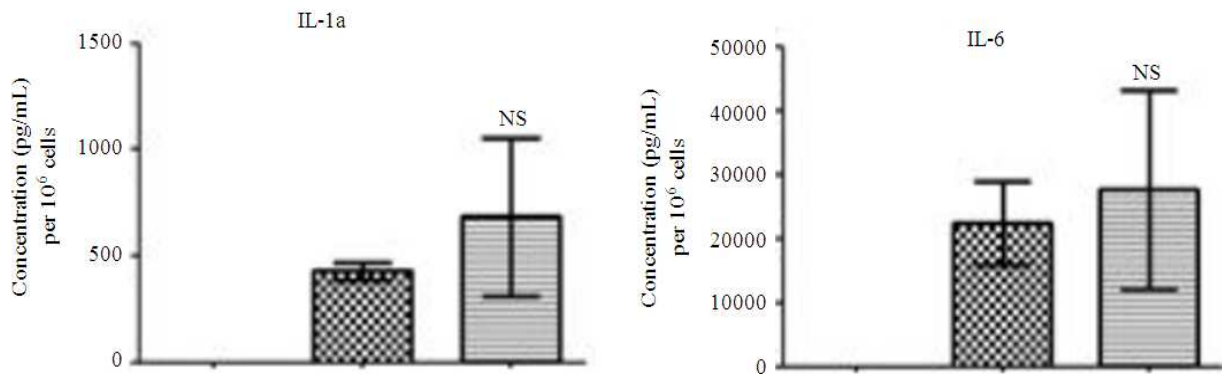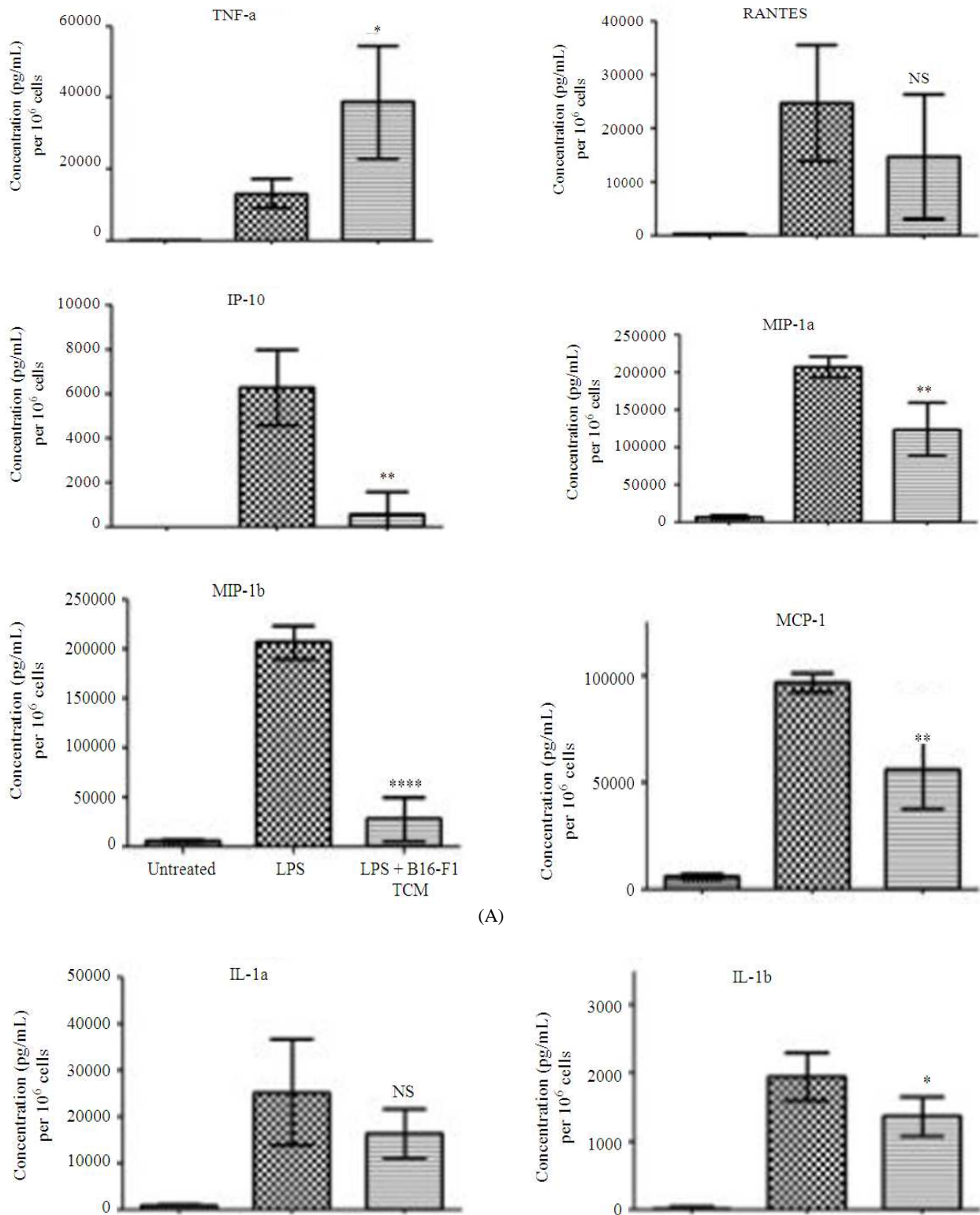
(A)

(B)

Fig. 2. LPS-induced cytokine/chemokine secretion by DC2.4 and JAWSII DC. DC2.4 and JAWSII cells were cultured for 24 h in normal growth media in the presence/absence of 1 µg mL^{-1} LPS. Following culture, supernatants were collected for analysis by ELISArray to detect expression of a panel of cytokines (A) and chemokines (B). Normalized data represent the O.D. 450-570 values of LPS-stimulated cells with the O.D. 450-570 values of untreated cells subtracted out.
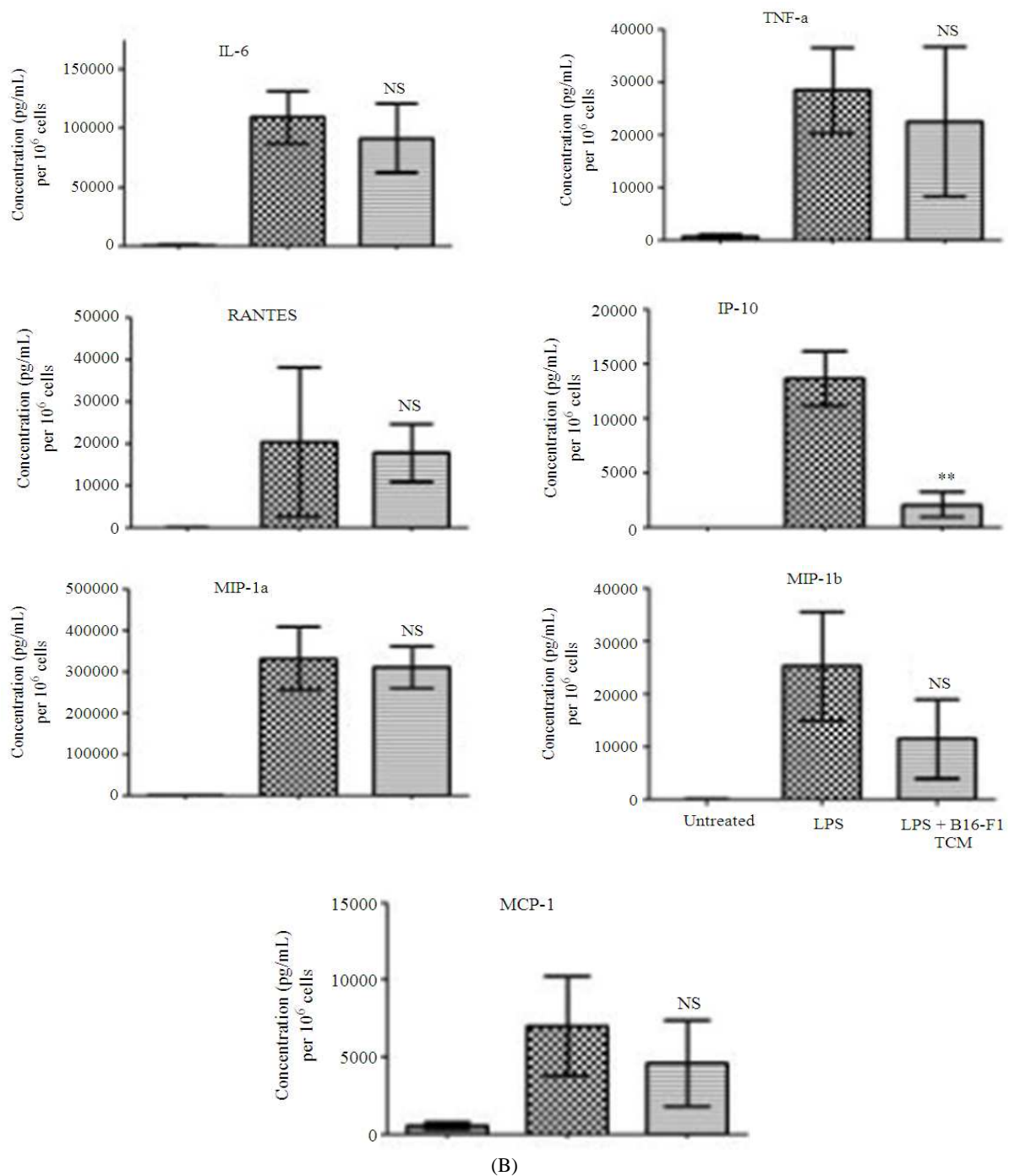
(A)

Fig. 3. B16-F1 tumor-altered cytokine/chemokine secretion by LPS-stimulated DC2.4 and JAWSII DC. DC2.4 (A) and JAWSII (B) cells were cultured for 24 h in normal growth media or in TCM derived from B16-F1 cultures in the presence/absence of 1 µg mL^{-1} LPS. Following culture, supernatants were collected for quantitative ELISA analysis of cytokines/chemokines identified previously by ELISArray. No cytokines/chemokines presented were detected in B16-F1 TCM alone. Data are expressed as the mean ± SD of 4 independent experiments

Importantly, all of the cytokines and chemokines detected in these experiments were produced by the DC lines; these molecules were not produced by B16-F1 melanoma cells as determined by ELISA analysis of TCM alone (data not shown). Collectively, these data demonstrate that B16-F1 melanoma-derived factors are capable of suppressing DC maturation and activation and that the DC2.4 and JAWSII dendritic cell lines are each effective *in vitro* models for studying melanoma-associated suppression of different cytokine/chemokine signaling pathways.

4. DISCUSSION

DC maturation and activation are critical processes that determine DC function and shape overall innate and adaptive immune responses within a host. Because of the central role played by DC in dictating the outcome of host immunity to a particular source of antigen, it is important to understand the factors that regulate their maturation and activation. In addition, although several mechanisms of tumor immune evasion have been described (Rabinovich *et al.*, 2007), the influence of tumors on DC maturation and activation and the role of DC in determining successful anti-tumor immunity versus tumor immune escape have yet to be extensively studied. In this report, we utilize an *in vitro* model system to evaluate the influence of B16-F1 melanoma-derived factors on the LPS-induced maturation and activation of two DC lines, DC2.4 and JAWSII. Both of these cells lines are relatively immature in their resting state, expressing low levels of MHC class II and costimulatory molecules and producing little, if any, of a number of cytokines and chemokines. Both cell lines are matured and activated by LPS stimulation, which upregulates expression of MHC class II, costimulatory molecules and proinflammatory cytokines and chemokines. We demonstrate that soluble tumor-derived factors from B16-F1 melanoma suppress the LPS-induced upregulation of costimulatory and MHC class II molecules on both the DC2.4 and JAWSII DC lines. Additionally, these tumor-derived factors alter LPS-induced secretion of several proinflammatory cytokines and chemokines by DC2.4 cells, while JAWSII cytokine/chemokine production, with the exception of IL-1β and IP-10, is unaffected by B16-F1-derived factors. Collectively, these results indicate that melanoma-derived factors can suppress dendritic cell maturation/activation and that the DC2.4 and JAWSII dendritic cell lines are each effective *in vitro* models for studying different aspects of this phenomenon. Furthermore, because of the role of DC in the induction of both innate and adaptive immunity, our data also

suggest that the interaction between DC and tumors is likely a critical factor in determining the overall quality of an anti-tumor immune response.

Anti-tumor immune responses are frequently detected in melanoma patients. In some cases, functional CD8^{+} T cell responses directed against melanocyte differentiation antigens correlate with tumor regression and enhanced survival (Rosenberg and White, 1996; Yamshchikov *et al.*, 2001). However, in many cases dysfunctional CD8^{+} T cells are recovered from tumors and tumor-draining lymph nodes of melanoma patients (Mortarini *et al.*, 2003; Anichini *et al.*, 2003; Zippelius *et al.*, 2004). It has recently been reported in a preclinical B16-F1 murine melanoma model that CD8^{+} T cell responses induced against the highly conserved tyrosinase-derived Tyr_{369} antigen (a well-characterized human tumor antigen) are also dysfunctional, exhibiting robust proliferation but minimal effector function (Hargadon *et al.*, 2006). The basis for this melanoma antigen-specific CD8^{+} T cell dysfunction in both patients and animals has remained unclear. While it is possible that tumors or tumor-derived factors directly suppress effector CD8^{+} T cell differentiation, it has been suggested that this T cell dysfunction might also arise from an influence of the tumor on cross-presenting DC (Hargadon *et al.*, 2006; Ferguson *et al.*, 2008). This latter possibility has been difficult to address by either *in vivo* or *ex vivo* analyses due to the limiting number of DC that can be isolated from melanoma patients or tumor-bearing animals. Surprisingly, despite the utility of DC lines as *in vitro* models for studying DC function, only one other group has examined the influence of a tumor on the maturation and activation of a DC line (Alshamsan *et al.*, 2010). In their study, melanoma-derived factors actually enhanced LPS-induced expression of select costimulatory molecules on DC2.4 cells, although cytokine and chemokine profiles were not assessed. Importantly, that previous study evaluated the effects of TCM derived from B16-F10, a different variant of B16 melanoma than the B16-F1 line that is associated with dysfunctional CD8^{+} T cell responses *in vivo* (Hargadon *et al.*, 2006) and that suppresses DC maturation and activation in our current study. Additionally, we have recently demonstrated that a chemically mutated variant of B16, the poorly tumorigenic D5.1G4 melanoma, is significantly less immunosuppressive than B16-F1 with respect to the influence of tumor-derived factors on DC2.4 maturation and activation (Hargadon *et al.*, 2012). We have also observed that B16-F1 secretes significantly greater amounts of the immunosuppressive cytokines TGFβ1 and VEGF-A than does D5.1G4 (unpublished data) and we are currently exploring the roles of these tumor-derived factors in the suppression of DC2.4

maturation and activation. It is therefore very likely that differences in these or other soluble factors derived from B16 melanoma variants account for the different effects of melanoma-derived factors on DC maturation and activation observed in our current and previous studies and the study by Alshamsan *et al.* (2010). Based on earlier studies that document the B16-F1-associated dysfunction of tumor Ag-specific CD8^{+} T cells (Hargadon *et al.*, 2006; Ferguson *et al.*, 2008) and our current observations that B16-F1-derived factors suppress the maturation and activation of DC2.4 and JAWSII cells, it will be interesting to determine whether these B16-F1-altered DC lines induce the type of incomplete CD8^{+} T cell differentiation associated with this tumor *in vivo*. We believe that this *in vitro* model offers a nice system for investigating the impact of tumor-altered DC on the quality of anti-tumor immune responses and we are currently exploring how these tumor-altered DC influence both CD8^{+} cytotoxic and CD4^{+} helper T cell activation and differentiation.

The LPS-induced secretion of a number of chemokines (RANTES, MCP-1, MIP-1α, MIP-1β and IP-10) by the DC2.4 and JAWSII DC lines is an intriguing finding. Interestingly, it has been shown that human monocyte-derived DC also produce IP-10 following LPS stimulation (Re and Strominger, 2001). IP-10 plays an important role in the chemoattraction of Th1 helper T cells, which express high levels of the CXCR3 receptor (Sallusto *et al.*, 1998). It is interesting to speculate that recruitment of such Th1 cells into a tumor is likely to improve the activation of tumor-infiltrating CD8^{+} T cells and that suppression of IP-10 production by tumor-associated DC might lead to a diminished anti-tumor T cell response. The suppressive activity of B16-F1-derived factors on LPS-induced IP-10 production by DC2.4 and JAWSII cells therefore has potentially significant implications for the quality of anti-tumor T cell responses. Additionally, we also observed significant suppression of MCP-1, MIP-1α and MIP-1β production by B16-F1-influenced DC2.4 cells and these chemokines regulate the migration of a number of immune cells that include monocytes, macrophages, NK cells and T cells (Olson and Ley, 2002). Melanoma-associated interference with the production of these chemokines by DC may therefore impact the recruitment of many immune effectors to the tumor and it will be interesting to compare in future studies the immune infiltration of B16-F1 melanoma with that of melanoma variants that do not suppress DC chemokine production as efficiently. Such a comparison will allow for the correlation of immune cell infiltration of a tumor with the efficacy of the anti-tumor immune response and may suggest strategies for targeting the particular immune effector types associated with greater tumor control to the tumor.

A variety of factors that are secreted by many tumors have been shown to interfere with DC function (Yang and Carbone, 2004; Fricke and Gabrilovich, 2006; Lin *et al.*, 2010) and melanomas in particular secrete a number of soluble factors that modulate immune responses (Ilkovitch and Lopez, 2008). B16-F1 melanoma secretes bioactive TGF-β1 (Peter *et al.*, 2001; our data not shown] and this immunosuppressive cytokine can inhibit costimulatory molecule expression as well as cytokine production by DC (Yamaguchi *et al.*, 1997; Geissmann *et al.*, 1999). B16-F1 also secretes VEGF-A but does not produce IL-10 (unpublished data) and it remains unknown whether this melanoma secretes other immunoregulatory factors. We are currently investigating the profile of immunosuppressive proteins secreted by B16-F1 so that we may test these molecules both individually and collectively for their role in the B16-F1-associated suppression of DC maturation and activation. Conversely, we also hope to gain mechanistic insights into B16-F1-induced DC immunosuppression by comparing the cell surface receptor expression of DC2.4 and JAWSII DC. Because JAWSII DC are less susceptible than DC2.4 cells to B16-F1-mediated suppression of chemokine production, it will be interesting to determine whether there are immunoregulatory receptors uniquely expressed by DC2.4 cells that increase their susceptibility to immunosuppressive factors. This information may shed light on the cellular targets of B16-F1-derived factors and ultimately point to the mechanism(s) by which this tumor suppresses DC function. Identification of the tumor-derived factors responsible for DC immunosuppression and their targets will suggest strategies for interfering with tumor-associated DC dysfunction that may improve the functional quality of endogenous DC in cancer patients as well as the efficacy of exogenous DC-based anti-tumor vaccines.

5. CONCLUSION

The DC lines DC2.4 and JAWSII are useful models for the study of factors that regulate DC maturation and activation. Tumor-derived factors from B16-F1 melanoma suppress the LPS-induced upregulation of costimulatory and MHC class II molecules on both of these DC lines. Conversely, LPS-induced proinflammatory cytokine and chemokine production by these two DC lines is differentially regulated by B16-F1-derived factors. Therefore, both the DC2.4 and JAWSII DC lines will serve as useful tools for future investigations of the mechanism of melanoma-associated suppression of DC maturation and activation as well as the basis for resistance versus susceptibility of DC to such immunosuppression.

6. ACKNOWLEDGEMENT

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