Human mesothelioma induces defects in dendritic cell numbers and antigen processing function which predict survival outcomes

Scott M.J. Cornwall, Matthew Wikstrom, Arthur W. Musk, John Alvarez, Anna K. Nowak, and Delia J. Nelson

a School of Biomedical Sciences, Immunology and Cancer Group, Curtin University, Perth, Western Australia (WA), 6102
b CHIRI Biosciences Research Precinct, Curtin University, Perth, WA, 6102
c University of WA, Lions Eye Institute, Nedlands. WA, 6009
d Department of Respiratory Medicine, Sir Charles Gairdner Hospital, Nedlands, WA 6009
e The Mount Hospital, 150 Mounts Bay Rd, Perth, WA 6000
f School of Medicine and Pharmacology, University of Western Australia. Nedlands, Perth, WA 6009
g Department of Medical Oncology, Sir Charles Gairdner Hospital, Nedlands, Perth, WA 6009

Accepted author version posted online: 31 Aug 2015.

To cite this article: Scott M.J. Cornwall, Matthew Wikstrom, Arthur W. Musk, John Alvarez, Anna K. Nowak & Delia J. Nelson (2015): Human mesothelioma induces defects in dendritic cell numbers and antigen processing function which predict survival outcomes, OncoImmunology, DOI: 10.1080/2162402X.2015.1082028

To link to this article: http://dx.doi.org/10.1080/2162402X.2015.1082028

Disclaimer: This is a version of an unedited manuscript that has been accepted for publication. As a service to authors and researchers we are providing this version of the accepted manuscript (AM). Copyediting, typesetting, and review of the resulting proof will be undertaken on this manuscript before final publication of the Version of Record (VoR). During production and pre-press, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal relate to this version also.

Taylor & Francis makes every effort to ensure the accuracy of all the information (the “Content”) contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any
Title: Human mesothelioma induces defects in dendritic cell numbers and antigen processing function which predict survival outcomes

Running title: Human mesothelioma induces defects in dendritic cell numbers and antigen processing function which predict survival

Authors: Scott M.J. Cornwall¹,², Matthew Wikstrom³, Arthur W. Musk⁴, John Alvarez⁵, Anna K. Nowak⁶,⁷ and Delia J. Nelson¹,².

Institutions:
¹School of Biomedical Sciences, Immunology and Cancer Group, Curtin University, Perth, Western Australia (WA), 6102.
²CHIRI Biosciences Research Precinct, Curtin University, Perth, WA, 6102.
³University of WA, Lions Eye institute, Nedlands, WA, 6009.
⁴Department of Respiratory Medicine, Sir Charles Gairdner Hospital, Nedlands, WA 6009.
⁵The Mount Hospital, 150 Mounts Bay Rd, Perth, WA 6000.
⁶School of Medicine and Pharmacology, University of Western Australia. Nedlands, Perth, WA 6009.
⁷Department of Medical Oncology, Sir Charles Gairdner Hospital, Nedlands, Perth, WA 6009.

Corresponding author:
Dr. Delia Nelson, c/o School of Biomedical Sciences, Curtin University, Kent St., Bentley, Perth, Western Australia, Australia, 6102.
Tel: 618 9266 9785; Fax: 618 9266 2342; E-mail: Delia.Nelson@curtin.edu.au
Abstract

Mesothelioma is an almost invariably fatal tumor with chemotherapy extending survival by a few months. One immunotherapeutic strategy is to target dendritic cells (DCs), key antigen presenting cells involved in antigen presentation, to induce antigen-specific T cell responses. However, DC-targeting will only be effective if DCs are fit-for-purpose, and the functional status of DCs in mesothelioma patients was not clear. We found that mesothelioma patients have significantly decreased numbers of circulating myeloid (m)DC1 cells, mDC2 cells and plasmacytoid (p)DCs relative to healthy age and gender-matched controls. Blood monocytes from patients could not differentiate into immature monocyte-derived DCs (MoDCs), indicated by a significantly reduced ability to process antigen and reduced expression of costimulatory (CD40, CD80 and CD86) and MHC (HLA-DR) molecules, relative to controls. Activation of mesothelioma-derived MoDCs with LPS+/−IFNγ generated partially mature MoDCs, evident by limited up-regulation of the maturation marker, CD83, and the costimulatory markers. Attempts to rescue mesothelioma-derived DC function using CD40Ligand(L) also failed, indicated by maintenance of antigen processing capacity and limited up-regulation of CD40, CD83, CD86 and HLA-DR. These data suggest that mesothelioma patients have significant numerical and functional DC defects and that their reduced capacity to process antigen and reduced expression of costimulatory molecules could induce anergized/tolerized T cells. Nonetheless, survival analyses revealed that individuals with mesothelioma and higher than median levels of mDC1s and/or whose MoDCs matured in response to LPS, IFNγ or CD40L lived longer, implying their selection for DC-targeting therapy could be promising especially if combined with another treatment modality.

Keywords: Mesothelioma, aging, immunotherapy, myeloid dendritic cells, plasmacytoid dendritic cells, CD40Ligand, survival outcomes
Introduction

Mesothelioma is an aggressive cancer of the mesothelium with a known carcinogen, asbestos, and a mean survival from diagnosis of only 9 months. However, some patients live substantially longer, including some who do not receive active therapy. Several prognostic markers have been identified and include epithelioid histology, normal haemoglobin, neutrophil-to-lymphocyte ratio and white cell counts, as well as younger age, with the latter two points implying a cancer-controlling role for the immune system. There is a lengthy latency period between asbestos exposure and detectable disease, which may reflect not only the time for a critical mutational load to develop, but also increasing age-related and tumor-induced immune dysfunction, eventually leading to tumor escape. Current treatment options including surgery and/or chemotherapy extend survival by only a few months. Therefore, research into other treatment strategies is warranted and immunotherapy has shown promise in mesothelioma.

One immunotherapeutic strategy is to target dendritic cells (DC). DCs are key antigen presenting cells that, in their immature state, take up and process antigens including tumor antigens, and upon appropriate maturation present them to naïve T cells to induce specific immune responses. DCs consist of a number of different yet functionally related subsets. Circulating DCs comprise myeloid and plasmacytoid (p)DCs. Myeloid (mDC) or conventional DCs are made up of at least two subsets and originate from CD34+ progenitors or CD14+ monocytes. The more common mDC1 cell is a major stimulator of T cells, and secretes IL-12 to induce polarization of naive CD4+ T cells into Th1 cells. The rarely occurring mDC2 cell type plays a role in cross presentation and shares many genetic similarities to cross-presenting CD8+ DCs in mice. Both mDC subpopulations are efficient in uptake, processing and presentation of antigens. Plasmacytoid DCs are generated from either CD34+ progenitors or CD11c+ blood precursors and rapidly secrete large amounts of interferon alpha (IFNα) in response to viral challenge. Monocyte-derived DCs (MoDCs) represent DCs that are rapidly generated in response to inflammation. Thus, dysfunction of one or more DC subsets
could result in aberrant immune responses.

Studies in other human cancers such as squamous cell carcinoma of the head and neck, prostate cancer, pancreatic cancer, breast cancer, Kaposi sarcoma and multiple myeloma have shown significant decreases in the numbers of circulating mDCs $^{19-21}$, pDCs $^{22}$, or mDCs and pDCs relative to healthy age-matched controls $^{23-26}$. Moreover, DCs extracted from tumors expressed an immature phenotype and were poor stimulators of T cell proliferation. $^{27}$ This may be explained by observations that blood DCs and MoDCs generated from patients with cancers such as breast cancer, hepatocellular carcinoma and squamous cell carcinomas of the head and neck expressed significantly lower levels of the antigen presenting molecule, HLA-DR, and/or low levels of the CD80 and CD86 co-stimulatory molecules $^{20,21,25,28}$ relative to DCs from healthy volunteers; these data imply an inability to appropriately activate T cells in cancer patients. Mouse models of mesothelioma have shown that even though tumor antigen presentation to CD8$^+$ T cells can be detected in draining lymph nodes, a function likely performed by DCs, the consequent T cell response was weak and unable to prevent tumor growth, implying inadequate co-stimulation. $^{29}$

There are no published data on the numbers and function of DCs in mesothelioma patients. We aimed to determine the effect of mesothelioma on pDC, mDC1 and mDC2 numbers in peripheral blood of patients compared to healthy aged and gender matched controls. The study also assessed the capacity for monocytes from both cohorts to differentiate into MoDCs, as well as their ability to process and present antigen and respond to microbial, cytokine and CD40 ligand (CD40L) stimulation. We identified profound deficits in DC numbers and function that may be important in the context of immunotherapy strategies. However, our data also suggests that functional DCs may contribute to survival in people with mesothelioma and that an immunotherapy tailored to improve DC numbers and function could improve patient outcomes as increased levels of mDC1s and surface CD80 in individual patients were associated with prolonged survival.
Results

Patient characteristics
48 people with mesothelioma and 40 age matched healthy volunteers were recruited to the study. Greater than 90% of participants with mesothelioma were newly diagnosed. The mean age of participants with mesothelioma was 66.9 years (range 47-84; SD 8.4) and for healthy volunteers was 67.5 years (range 48-84, SD 8.3, p = 0.7). 10 people with mesothelioma (21%) and 15 healthy volunteers (37%) were female (p = 0.17).

Mesothelioma patients have decreased numbers of blood DC subsets
Circulating DC subsets in mesothelioma patients versus age-matched controls were examined using flow cytometry. Gating eliminated debris, red blood cells, B-cells, monocytes and granulocytes (Figures 1a and 1b). DC sub-populations were identified by high expression of BDCA-1 (CD1c; mDC1s, Figures 1c and 1e), very high expression of BDCA-3 (CD141; mDC2s, Figures 1d and 1f) and high expression of BDCA-2 (CD303; pDCs, Figures 1c and 1g).

Myeloid DC1, mDC2 and pDC subsets in healthy individuals showed an age-related decrease in numbers, with pDCs demonstrating a statistically significant decrease (p < 0.0001, Fig 1 e-g). The numbers of all three DC subsets were even further reduced in mesothelioma patients relative to the age-matched controls with differences between patients and controls being statistically significant (p < 0.0001). These data suggest that the immune system in mesothelioma patients may be impaired relative to healthy age-matched controls.

Mesothelioma-derived monocytes differentiate into immature CD14+ MoDCs
We next examined whether reduced numbers of circulating DCs in mesothelioma patients were associated with changes in DC function. As direct functional analysis of blood DC subsets is difficult due to low numbers this series of experiments involved the generation of MoDCs from monocyte precursors in vitro. Monocytes from mesothelioma patients and healthy controls were exposed to GM-CSF and IL-4 (Figure
2a) and their ability to differentiate into immature (i)MoDCs investigated by examining expression of key DC surface markers on gated large, CD14\(^{-}\) cells. No differences were observed between mesothelioma patients and age-matched controls in the percentage of CD14\(^{-}\) cells that differentiated into classical CD11c\(^{+}\) DCs, or in CD11c surface expression levels (MFIs) (Figures 2b,c,d and e).

CD40 and CD86 are important co-stimulatory molecules and CD83 is a key maturation marker. No differences were seen in the percentage of cells expressing CD40, CD86, or CD83 (Figures 2g,j,m). No differences were seen for surface expression levels (MFI) of CD86 and CD83 (Figure 2k and n) in mesothelioma versus healthy controls. However, a statistically significant decrease was observed in mesothelioma patients for CD40 expression levels (Figure 2h: \(p = 0.04\)). Overall, these data imply that mesothelioma-derived monocytes can differentiate into immature CD14\(^{-}\) MoDCs however, lower CD40 expression may interfere with their ability to be fully activated.

*Mesothelioma-derived monocytes can differentiate into immature CD11c\(^{+}\) CD1a\(^{+}\) DCs*

CD11c\(^{+}\) DCs can differentiate into CD11c\(^{-}\)CD1a\(^{-}\) DCs or CD11c\(^{+}\)CD1a\(^{-}\) DCs that play a pro-inflammatory or anti-inflammatory role respectively \(^30\); the latter may prevent T cell activation. Therefore, iMoDCs were further gated as CD11c\(^{+}\) cells (Supplementary Figures 1a and b) then as CD1a\(^{+}\)CD11c\(^{+}\) DCs (Supplementary Figure 1c). No differences were observed between MoDCs from mesothelioma patients and controls in either the percentage of CD11c\(^{+}\) cells expressing CD1a or CD1a expression levels (Supplementary Figures 1d and e). CD1a\(^{+}\)CD11c\(^{+}\) DCs were further examined for expression of HLA-DR (a MHC class II molecule involved in antigen presentation to CD4\(^{+}\) T cells) and CD80 (a co-stimulatory molecule). Again, no differences were seen for the percentage of cells expressing HLA-DR (>99% were HLA-DR\(^{+}\)), CD80 (Supplementary Figure 1f), or for expression levels of HLA-DR (Supplementary Figure 1h) and CD80 (Supplementary Figure 1g). These data suggest that mesothelioma DCs maintain their ability to develop into pro-inflammatory CD11c\(^{+}\)CD1a\(^{+}\) DCs.

*Immature MoDCs from mesothelioma patients cannot process antigen*
The primary role of immature DCs is to take up and process antigen. The DQ-OVA assay was used to compare the antigen processing ability of MoDCs from mesothelioma patients versus healthy volunteers. Whilst no differences were observed for the percentage of MoDCs able to process antigen (Figures 3a-c), mesothelioma patients demonstrated significantly lower levels of antigen processing (MFIs) relative to healthy controls (Figure 3d: \( p = 0.004 \)). These data reveal an important defect in antigen processing ability in DCs from mesothelioma patients.

**MoDCs from mesothelioma patients cannot fully up-regulate CD40 and CD86 following activation**

DCs need to be appropriately matured and express key antigen presenting and co-stimulatory molecules before they can induce functional T cell responses. Their ability to respond to factors that activate DCs may also reveal a potential therapeutic approach. Therefore, we compared the maturational response of iMoDCs from mesothelioma patients and healthy volunteers to the microbial component lipopolysaccharide (LPS) with or without IFN\( \gamma \), or to CD40L for 48 hours. CD40L was included as CD40-targeting strategies are already available for cancer patients. No differences were seen between the percentage of MoDCs from mesothelioma patients and controls expressing CD11c, CD83, CD40 or CD86 after activation (Supplementary Figures 2a-d). With the exception of response to IFN\( \gamma \) maturation, there was a trend towards decreased expression levels of CD11c (Figure 4b), CD83 (Figure 4c), CD40 (Figure 4d) in MoDCs from mesothelioma patients in response to each maturation stimulus. The only statistically significant decrease was seen for CD86 on mesothelioma MoDCs activated with LPS relative to age-matched controls (Figure 4e: \( p = 0.04 \)).

There were no differences in the percentage of CD11c\(^+\) cells co-expressing CD1a (Supplementary Figure 3a) or CD1a expression levels (Supplementary Figure 4a). No differences between patients and controls were noted in the percentage of CD1a\(^+\)CD11c\(^+\) DCs expressing CD80 or HLA-DR (Supplementary Figures 3b and c), although again, a trend towards decreased expression levels of CD80 and HLA-DR (Supplementary Figure 4b and c) was seen.
In general, LPS, with or without IFNγ, was the best inducer of likely beneficial phenotypic changes, whilst IFNγ and CD40L were the weakest. Taken together, these data imply that mesothelioma MoDCs do not achieve full activation relative to MoDCs from healthy controls.

**MoDCs from mesothelioma patients lose antigen-processing function after activation**

We further examined whether MoDCs from mesothelioma patients appropriately matured in response to stimuli by losing their capacity to process antigen. The healthy, age-matched (elderly) controls did not fully lose their antigen processing ability after LPS +/- IFNγ stimulation with > 45% of matured MoDCs retaining their antigen processing ability (Figure 5a), indicating an age-related defect (manuscript in preparation). In contrast, > 75% of mesothelioma-derived LPS +/- IFNγ-matured MoDCs lost their ability to process antigen, this was matched by lower expression levels of degraded FITC-DQ-OVA indicating a low capacity to process antigen (Figures 5a and b). These data suggest that mesothelioma-derived MoDCs mature by loss of antigen processing better than their healthy counterparts. However, it should be noted that immature DCs from mesothelioma patients had a weaker baseline antigen processing capacity (Figure 3d, 5b).

**Mesothelioma MoDCs maintain their ability to induce T cell proliferation**

The ability of DCs to present antigen to T cells increases with activation/maturation. Therefore, activated matured MoDCs from mesothelioma patients and healthy controls were examined for their ability to induce proliferation of CFSE-labeled T cells from a universal healthy, male donor aged 34 years. CD4 and CD8 T cells were identified by size and surface marker expression. No differences were noted in either CD4+ or CD8+ T cell proliferation induced by MoDCs from mesothelioma patients compared to healthy controls (Figures 6a-d). LPS, with or without IFNγ, proved to be the most potent stimulus, and CD40L the weakest, in terms of inducing T cell proliferation. These data show that MoDCs from mesothelioma patients are not defective in their ability to induce T cell proliferation.
Mesothelioma MoDCs secrete similar levels of cytokines in response to activation

Cytokines produced by DCs after activation determine the immune response that will be ultimately generated, therefore culture media from activated MoDCs was analyzed by CBA. Regardless of the stimulus used, MoDCs from mesothelioma patients secreted similar levels of the suppressive cytokines IL-10 and VEGF (Figures 7a and b), as well as the pro-inflammatory cytokines TNF, IL-12p70 and IFNγ to their healthy counterparts (Figures 7c-d). These data show that MoDCs from mesothelioma patients are not defective in their cytokine responses. The dominating cytokines were IFNγ and IL-10 in response to LPS; IFNγ, IL-12p70 and TNF in response to LPS/IFNγ. CD40L and IFNγ were poor inducers of cytokine secretion.

Increased circulating mDC1 numbers are associated with prolonged survival in mesothelioma patients

To investigate whether numbers of circulating DCs seen in people with mesothelioma reflected patient outcomes, patients were ranked and dichotomised into those above and below the median number of pDCs, mDC1s or mDC2s, and Kaplan-Meier plots generated. Patients with a higher than median number of circulating mDC1s demonstrated a significant increase in survival (Figure 8a; p = 0.02). In contrast, higher pDCs or mDC2 numbers did not predict for longer survival (data not shown).

Mesothelioma patients with MoDCs that maintain some ability to respond to maturation stimuli live longer

Loss of antigen processing ability by immature DCs in response to activation stimuli is evidence of DC maturation, and patients with lower than median antigen processing capacity after stimulation with LPS and IFNγ lived longer, with a median survival of 44 months versus > 55 months. (Figure 8b; p = 0.01). Expression of key activation surface markers post stimulation with LPS +/- IFNγ or CD40L were also correlated against survival time. Only patients whose CD40L or IFNγ-matured MoDCs demonstrated a higher than median expression of CD80 experienced a significantly increased survival time (Figure 9c; p = 0.04 and p = 0.045 respectively). Taken together, these data imply
that patients with sufficient numbers of mDC1 and/or MoDCs that mature in response to stimuli experience a longer survival duration.

Discussion
Our study revealed that aging might impact on the immune system in people with mesothelioma, a cancer that is predominantly seen in the elderly due to the long latency between asbestos exposure and development of disease. Changes to circulating DC subsets and DCs derived from precursor monocyte cells were examined. We showed that pDC numbers were significantly reduced in the age group in which mesothelioma starts to emerge. However, we also showed that whilst the aging process likely contributes to reducing numbers of circulating pDCs, mesothelioma amplified this effect and also reduced the numbers of mDC1 and mDC2 cells. In regards to the latter finding, similar results have been reported in breast carcinoma, multiple myeloma, Kaposi’s sarcoma and pancreatic adenocarcinoma. It is not yet clear if reduced circulating DC numbers reflect events occurring in tumors, regional lymph nodes or even in the bone marrow. One possibility is that tumor-associated factors interfere with the ability of bone marrow precursors to differentiate into DC subsets before they enter the bloodstream. Alternatively, tumor-associated DCs may lose their migratory capacity, or undergo apoptosis. Further studies are required, but candidate tumor-derived factors which may perturb DC differentiation or function include TGFβ, VEGF and IL-6, all of which are found in high concentration in pleural effusions associated with mesothelioma. Loss of DC numbers and function will impair background anti-tumor immunity and the efficacy of subsequent anti-tumor immunotherapy, as shown by DC depletion studies in mice.

An important and novel observation found in these studies was that not only were numbers lower for all DC subsets in people with mesothelioma, but these lower numbers also predicted for shorter survival, at least for mDC1. These data raise the hypothesis that mDC1 cells contribute to patient survival likely via promoting protective immunity. This is supported by further data showing that patients whose MoDCs maintained their ability to increase CD80 expression in response to activation stimuli demonstrated...
increased survival. Interestingly, others have shown that increased frequency of mDC1 after sunitinib treatment is predictive for tumor regression and improved progression-free survival in renal cancer patients. This is important because of the increasing role of immunotherapy in the treatment of cancer, and the potential future application of this therapy to mesothelioma. Moreover, the data raise the possibility that targeting mDC1s or MoDCs using immunotherapy might prolong survival. These data also imply that there is a subgroup of mesothelioma patients whose DCs are not fully impaired and treating them with immunotherapy may extend survival duration. The use of immunotherapy is supported by murine studies that have demonstrated its potential for mesothelioma as a stand-alone therapy, or in combination with chemotherapy and/or surgical debulking of tumors. The recent report of successful checkpoint blockade in mesothelioma suggests the possibility of combination of checkpoint blockade with strategies targeting impairment in DCs.

The most concerning effect of mesothelioma was loss of antigen processing function in immature MoDCs. This is important as the data show that even in an environment removed from tumor-derived suppressive factors, iMoDCs from mesothelioma patients were unable to process antigen to the same levels as their healthy counterparts, implying irreversible mesothelioma tumor-induced dysfunction. Antigen processing is a key function of immature DCs, including those in the tumor microenvironment, as these cells are critically required to take up tumor antigen for presentation to T cells in draining lymph nodes. Loss of antigen processing function was clearly mesothelioma-specific and independent of age, as iMoDCs from healthy aged-matched individuals maintained this ability. Again, this loss of function could have been driven by tumor-associated factors irreversibly affecting monocytic precursors. Similar results have been reported for late stage breast cancer patients. If this effect is exerted upon tissue and circulating DC subsets, low levels of antigen processing might prevent efficient antigen presentation to T cells following DC maturation. This is supported by murine studies showing defects in T cell function in mice with mesothelioma, wherein tumor antigen-specific T cells either accumulate in draining lymph nodes, or leave lymph nodes but, without exogenous help, cannot prevent tumor progression. These data suggest that
identifying ways of rescuing antigen-processing function when tumor antigen-based vaccines are used might be an important immunotherapeutic strategy. Furthermore, given the clinical success of PD1 blockade and anti-CTLA4 blockade, rescuing DC function may be critical to the development of a CD8^+ T cell response which can be released from inhibitory feedback.

In healthy young adults, loss of antigen processing function is associated with DC maturation as they transition to a cell that presents antigen in MHC molecules to T cells. However, our data showed that healthy, elderly-derived MoDCs retained some capacity to process antigen following activation with LPS and/or IFNγ, implying an age-related inability to mature in a manner similar to young healthy controls. The mechanisms underlying this observation are unknown, but examining ROS within DCs may be useful, as studies of aged mice have shown that oxidative stress induces inadequate clearing of ROS (a hallmark of aging) leading to defects in antigen processing. Mesothelioma-derived iMoDCs demonstrated lower baseline levels of antigen processing ability which further reduced in response to LPS and/or IFNγ, reduced implying some level of maturation, however this response did not correlate with an increase in the maturation marker, CD83. It is unclear whether this is due to the presence of ROS, or to defects in the mechanisms involved in down-regulating antigen processing function such as lower levels of TAP proteins, similar to that observed in cancer patients, or if elderly-derived MoDCs adopt a macrophage-like function and maintain their ability to process and destroy pathogens. Importantly, patients with a higher than median ability to down-regulate antigen processing following activation with LPS and IFNγ lived significantly longer. This suggests that when the normal process of down-regulation of antigen processing is retained in mesothelioma patient DCs, they mature appropriately and present tumor antigens to T cells to induce activated T cells that may slow tumor progression. Furthermore, patients maintaining DCs with an ability to mature once they have processed antigen are more likely to respond to vaccine-based immunotherapy. Therefore, screening maturation responses may be a potential predictive tool for this approach.
Whilst mesothelioma-derived monocyte precursors responded to GM-CSF and IL-4 by differentiating into iMoDCs, including pro-inflammatory CD11c+CD1a+ DCs, there was evidence of functional impairment as they expressed lower baseline levels of costimulatory molecules, in particular CD40, which may interfere with their ability to be activated by therapeutic CD40-targetting strategies. This is supported by experiments attempting to activate mesothelioma-derived iMoDCs with LPS +/- IFNγ, or CD40L which revealed an inability to achieve full maturation relative to their healthy age-matched controls. A trend towards decreased expression of CD11c, CD83, CD40, CD80 and HLA-DR, with a significant decrease of CD86, was seen; all of which interfere with their ability to activate T cells. Similar deficiencies in expression of molecules involved in antigen presentation have been reported in late stage breast cancer, squamous cell carcinomas and hepatocellular carcinoma. Nonetheless, MoDCs from mesothelioma patients could induce allogeneic T cells to proliferate; it is possible that testing with antigen-specific T cells, such as those specific to tetanus toxoid, may have revealed greater differences between controls and patients. Our data contrasts with breast cancer studies showing that patient MoDCs had a decreased ability to induce allogeneic T cell proliferation. These data imply differences between cancer types. The type of T cell generated is crucial as immunotherapeutic strategies aim to induce effector and not regulatory T cells. Further studies are required to examine the type of T cell generated by mesothelioma-derived DCs.

DCs respond to activation by secreting cytokines. Our data show that MoDCs from mesothelioma patients are not defective in their cytokine responses. The cytokine response was determined by the stimulus used, with LPS/IFNγ inducing the strongest cytokine response from patients and controls alike. The dominating cytokines were IFNγ and TNF in response to LPS/IFNγ. Increased IFNγ and TNF indicate potential induction of a strong cell mediated anti-mesothelioma immune response. However, whilst DCs from mesothelioma patients trended towards higher levels of secretion of three immune-stimulatory cytokines (including IL-12p70) in response to LPS/IFNγ, this coincided with a trend towards lower surface CD40 levels relative to healthy controls. One possible explanation is that as patient DCs also trended towards secreting higher levels of IL-10
and VEGF in response to LPS/IFNγ, these factors may reduce the impact of the immune-stimulatory cytokines on CD40 expression. Indeed, CD40 expression is reported to be lower on blood DCs from cancer patients and blocking all isoforms of VEGF in these patients restored CD40 expression. LPS alone induced a mixed pro-inflammatory (IFNγ) and anti-inflammatory (IL-10 and VEGF) response which may thwart an anti-tumor immune response. These data are similar to studies in patients with Kaposi’s sarcoma, a cancer associated with immune suppression, in which LPS induced a decrease in DC-derived IL-12 alongside an increase in IL-10 indicating a failure in the immune system’s ability to keep the sarcoma in check. Both CD40L and IFNγ were poor inducers of cytokine secretion. This cytokine milieu may be critical in driving T cell responses, even with lower expression of co-stimulatory molecules. Thus, choice of stimulus may be critical when designing a DC-targeting immunotherapy for mesothelioma. Our data show that the combination of LPS and IFNγ may be sufficient to induce a strong anti-tumor response whilst use of LPS or IFNγ alone, or CD40L, may only offer a limited benefit unless combined with another treatment modality. Interestingly, patients with a higher than median percentage of MoDCs expressing CD80 following CD40L activation lived longer. These data suggest that the co-stimulatory molecule CD80 may play an important role in increasing survival, and that therapies aiming to stimulate increased expression of CD80 should be further investigated.

Strengths of this study include use of age and gender matched controls, immediate processing of fresh samples to avoid confounding factors introduced by freezing, plus use of a comprehensive panel to test DC attributes. The study had a small sample size however was adequately powered a priori to identify the primary endpoint of difference in DC numbers between patients and healthy controls. Differences in survival were identified on univariate analysis, and are provocative and hypothesis generating. We acknowledge that multivariate analysis incorporating other important prognostic factors such as histology and clinical status may be important, and that there may be confounding factors such as time from diagnosis. However our sample size was not sufficient to perform multivariate analysis. We also relied on iMoDCs given the extremely low DC numbers in mesothelioma patients. MoDCs are an increasingly
controversial cell type in regards to DCs, however recent studies have shown that murine bone marrow cells cultured with GM-CSF generate a heterogeneous group of CD11c+ MHC class II+ DCs that comprise conventional DCs and monocyte-derived macrophages, with both undergoing maturation upon LPS stimulation but responding differentially and remaining as separable entities. 57 This may also be true for GM-CSF/IL-4-driven blood-derived human cells, therefore CD14+ MoDCs remain useful to reveal differences between DCs from healthy elderly controls and cancer patients, both of whom are difficult groups to collect large blood volumes or perform serial sampling. Taken together, these data imply that mesothelioma affects DC maturation, likely through secretion of soluble factors. However, CD40L proved to be a weak DC stimulator in healthy, elderly control DCs, as well as in mesothelioma-derived DCs, relative to stimulation with LPS/IFNγ suggesting an age-related defect as well as a mesothelioma-driven one. These findings are important as CD40 activating strategies are being clinically tested in mesothelioma and other cancers; 58,59 low levels of CD40 expression and a poor response to CD40 ligation in people with mesothelioma may make CD40 activation a less attractive immunotherapy intervention. Future studies should look for expression of checkpoint inhibitory molecules on DCs, as high expression levels of the ligand for the checkpoint inhibitor, programmed cell death-1 (PD-1), on DCs in elderly hosts has been reported; 60 if this is true for DCs from mesothelioma patients then checkpoint blockade, which is in current clinical use in other indications and is undergoing clinical trials in mesothelioma, 61, could prove to be beneficial.

In conclusion, these studies demonstrated that a greater understanding is required of the effects of age and mesothelioma on DC subsets. We found specific age-related changes, in particular decreased circulating pDC numbers with further defects seen in people with mesothelioma including decreased circulating mDCs, decreased antigen processing in immature DCs, reduced expression of surface CD40 and a subsequent poor response to CD40 activation. Importantly, mesothelioma patients with higher numbers of circulating mDC1s and/or DCs that maintained one or more response to maturation signals lived longer. These studies show the importance of incorporating age-matched controls in
order to distinguish effects of cancer from effects of aging. These data also suggest that functional DCs may contribute to survival in people with mesothelioma and that an immunotherapy tailored to improve DC numbers and function, particularly antigen processing, prior to DC maturation, could improve patient outcomes and may be important to include in combination immunotherapies. Moreover, our data suggests that immunotherapies involving DCs, such as cancer vaccines, could utilize a screening protocol to select for patients with functional DC.

**Patients/Materials & Methods**

**Study subjects**
Forty-eight people with mesothelioma were recruited for this study by three clinicians, with three additional patients recruited through radio advertising. Patients were excluded from the study if they had undergone active anticancer treatment (chemotherapy, radiotherapy, surgery) in the previous 9 months. Forty age-matched healthy volunteers were by recruited by (i) radio advertising, (ii) print advertising in an elderly demographic newspaper, (iii) poster advertising, and (iv) recruitment from laboratory volunteers. Healthy volunteers were excluded from the study if they currently had cancer, autoimmune disorders or other severe immune disorders. Written informed consent was obtained prior to blood collection. Health status was determined using a study-specific survey. This study was approved by the Human Ethics Committees for Sir Charles Gairdner Hospital (#2008-041); the Mount Hospital (#EC50.1), and Curtin University (#HR68/2008).

**Collection of blood samples**
Fifty mL of whole anti-coagulated blood was collected via mid-arm venepuncture into five 10 mL K$_2$EDTA vacutainer tubes (BD Pharmingen, USA) and transported to the laboratory for immediate processing.

**Enumeration of DC subsets from whole blood**
DC subsets were quantified using a Blood Dendritic Cell Enumeration Kit (Miltenyi-Biotec, Germany) as per manufacturer's instructions. Briefly, 300 µl of whole blood was
incubated with 10 μl of an antibody cocktail containing BDCA1-PE (anti-human CD1c, clone AD5-8E7), BDCA2-FITC (anti-human CD303, clone AC144), BDCA3-APC (anti-human CD141, clone AD5-14H12), CD19-PeCy5 (B cells) and CD14-PeCy5 (monocytes) plus 5 μl of a dead cell discriminator (a fluorescent photolytic dye). The isotype control antibody cocktail containing mouse IgG2a-PE, IgG1-FITC, IgG1-APC, CD19-PeCy5 and CD14-PeCy5 and the dead cell discriminator were added to a control tube. All tubes were incubated horizontally for 10 min on ice under a 60W globe to illuminate the dead cell discriminator with visible light which binds covalently and irreversibly to nucleic acids of dead cells. Red blood cells were lysed (1.55 M NH₄Cl, 0.1 M KHCO₃, 1 mM EDTA, pH 7.4) at room temperature for 10 min in the dark and washing twice in FACS Buffer (1x PBS containing 1 % BSA (Sigma-Aldrich, USA), 2 % FCS (Thermo Fisher Scientific, USA) and 0.01 % sodium azide) the cell pellet was resuspended in 300 μl of FACS buffer, 150 μl of Fix Solution (3.7 % formaldehyde in PBS) and 5 μl Discriminator Stop Reagent (Deoxyribonucleic acid in 10 mM TRIS, 10 mM NaCl, 1 mM EDTA, pH8). Samples were analyzed on a FACSCantoII using Diva software (BD Biosciences). Absolute cell counts were determined by multiplying the number of DCs in the leukocyte gate (Figure 1) by the total number of PBMC in 50 mLs determined by staining with trypan blue solution using a haemocytometer.

Isolation of peripheral blood mononuclear cells (PBMCs)

Fifteen to 20 mL blood was aliquoted into 50 mL tubes and PBS (Invitrogen, USA) containing 2 mM EDTA (Sigma-Aldrich, USA) added to make a total volume of 35 mL per tube. The diluted blood was layered over 15 mL of Ficoll-paque PLUS (GE Healthcare, Sweden) and centrifuged at 400 g for 40 min at 20° C with the brake off. The interphase containing lymphocytes, monocytes and thrombocytes was removed and resuspended in 50 mL of PBS/2 mM EDTA. After centrifugation at 300 g for 10 min the cells were resuspended in 2 mM EDTA and centrifuged at 200 g for 10 min at 20° C twice to remove platelets.

In-vitro generation of Monocyte-derived DCs
Dendritic cells were prepared using a modified procedure. Briefly, 1 x 10^8 PBMCs were allowed to adhere to a 75 cm^2 tissue culture flask in RPMI (Invitrogen, USA) media containing 10 % FCS, 50 μM 2ME (Sigma-Aldrich, USA), 100 U/mL Penicillin and 50 mg/mL Gentamycin. Following a two hour incubation at 37º C in 5 % CO_2, non-adherent cells were removed and the remaining adherent cells (monocytes) cultured for 7 days in 80 ng/mL GM-CSF (Shenandoah, USA) and 10 ng/mL IL-4 (R&D Systems, USA) added on day 0 and supplemented on day 4. The MoDC cultures continuously contained 10 μg/mL Polymixin-B (Sigma-Aldrich, USA) to inactivate lipopolysaccharide (LPS).

**Lymphocyte isolation**

Peripheral blood mononuclear cells (1 x 10^8 cells) were left to adhere to a 75 cm^2 tissue culture flask in RPMI media containing 10 % FCS, 50 μM 2ME, 100 U/mL Penicillin and 50 mg/mL Gentamycin. Following a two hour incubation at 37º C, 5 % CO_2, the non-adherent cells predominantly lymphocyte population were collected for use as responder lymphocytes in the mixed lymphocyte reaction (MLR).

**MoDC stimulation**

On day 7 non-adherent immature MoDCs were washed twice by centrifugation at 1200 rpm for 5 minutes, placed into 75 cm^2 flasks, topped with 15 mL culture media, 80 ng/mL GM-CSF and 10 ng/mL IL-4. MoDCs were stimulated for 2 days with either 10 μg/mL LPS (Sigma-Aldrich, USA), 20 ng/mL IFN_γ (Sigma-Aldrich, USA), 10 μg/mL LPS and 20 ng/mL IFN_γ, or 0.66 μg/mL CD40L (Genscript, USA). One flask was used as a no-stimuli control containing 10 μg/mL Polymixin-B (Sigma-Aldrich, USA).

**DC phenotyping**

Cells were stained for CD1a-PeCy5 (clone HI149), CD11c-APC (clone B-ly6), CD14-FITC (clone M5E2), CD40-PeCy5 (clone 5C3), CD80-PE (clone 2D10), CD83-APC (clone HB15E), CD86-PE (clone 2331 (FUN-1)) and HLA-DR-APC-Cy7 (clone L242), all purchased from BD Pharmingen, USA. Cells were incubated for 30 min at 4º C in the
dark, washed in PBS and cell surface expression measured by flow cytometry using a BD FACSCanto II and Diva or Flow Jo (Tree Star Inc.) software.

**Antigen uptake and processing assay**

MoDCs (5 x 10^4 cells) in 100 μL of media were incubated with 1 μL of 1 mg/mL DQ-conjugated ovalbumin (DQ-OVA; Invitrogen) for 1 h at 37°C. Controls included cells incubated with DQ-OVA at 4°C and without DQ-OVA at 37°C and 4°C. Following washing, cells were analyzed by flow cytometry. MoDCs were gated for by size then for degradation of FITC-labeled DQ-Ovalbumin (DQ-OVA), which indicates antigen processing. Antigen uptake and processing was calculated as follows: 

\[
\text{[(Median Fluorescence intensity (MFI) of 37°C with DQ-OVA) – (MFI of 37°C without DQ-OVA)]} = \text{[(MFI of 4°C with DQ-OVA) – (MFI of 4°C without DQ-OVA)]}
\]

An increase in the calculated MFI corresponds to an increase in antigen uptake and processing ability.

**CFSE labeling T-cells**

Following incubation of PBMCs for two hours, the non-adherent population containing monocyte-depleted T cell-enriched cells was collected, washed in PBS and resuspended at 2 x 10^7 cells/mL in RPMI media containing 3.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich, USA), a fluorescent dye that binds to cell membranes. Cells were incubated at room temperature for 10 min, washed three times with RPMI and used as responder T cells for the MLR.

**The mixed lymphocyte reaction (MLR)**

MoDCs were seeded in duplicate into a 96 well plate at concentrations ranging from 1 x 10^3 to 1 x 10^5 cells/mL and 2 x 10^5 CFSE-labeled allogeneic T cells, from a universal, 34 year old male donor, added to each well. Control wells contained MoDCs alone, T cells alone and T cells stimulated with Concanavalin A (Con A; Sigma-Aldrich, USA). Plates were incubated in the dark at 37°C 5% CO₂ for 8 days. The cells were then washed and stained for CD4 and CD8 expression using CD4-PE (clone RPA-T4) and CD8-APC (clone RPA-T8; BD Pharmingen, USA), for 30 min at 4°C in the dark. Cells were
washed for analysis by flow cytometry. The parent T-cell population was identified based on the CFSE staining intensity of the T cells alone control. As T cells proliferate each new daughter generation contains half the CFSE of the previous generation resulting in a sequential halving of CFSE staining intensity, represented as individual peaks during flow cytometry, as we have published. The percentage of T cells proliferating was calculated by gating all CFSE+ cells and excluding the non-proliferating parent peak.

**Cytokine Bead Array (CBA)**
The cytokines TNFα, IL-10, IL-12(p70), VEGF and IFNγ were measured simultaneously by CBA (BD Pharmingen, USA) as per the manufacturer’s protocol.

**Statistics**
A sample size of 40 patients and 40 healthy controls was planned, as this was sufficient to detect a mean difference in DC cell count between patients and healthy controls of 20%, with a standard deviation of 25% of baseline and setting $\hat{U} = 0.0125$ (due to multiple comparisons) and $\hat{b} = 0.80$, allowing for 10% loss to follow up.

Statistical analyses were conducted using GraphPad Prism v4.03 (USA). Statistical differences were determined by a two-tailed Mann-Witney t-test and linear regression of continuous data. P-values less than 0.05 were considered statistically significant. Overall survival was considered from the date of study entry to the date of death. Participants who were still alive were censored at the date of their last confirmed clinical appointment. Survival analyses were performed using the Log-Rank test and displayed on a Kaplan Meier plot.

**Acknowledgments:** We thank the Slater and Gordon Asbestos Research Fund for supporting this project. The authors acknowledge the provision of research facilities and the scientific and technical assistance of the staff of CHIRI Biosciences Research Precinct core facility, Curtin University.
References


Figure 1: Mesothelioma patients have decreased numbers of blood DC subsets

Whole blood was stained and analysed by flow cytometry. Representative dot plot (a) showing gating of leukocytes by size (FSC) and granularity (SSC). CD14+ monocytes, granulocytes and CD19+ B-cells were further excluded by gating (b). Blood DC subsets were identified by high expression of BDCA-1 (c: mDC1), BDCA-3 (d: mDC2 and BDCA2 (c: pDC). Circulating mDC1 (e), and mDC2 (f) and pDCs (g) are shown as the number of DCs per mL of blood. Each dot represents an individual volunteer (mesothelioma: n = 48, age matched controls: n = 36). ***p < 0.0001.
Figure 2: Mesothelioma-derived monocytes differentiate into iMoDCs

Blood monocytes from mesothelioma patients and age-matched volunteers were differentiated into iMoDCs using GM-CSF and IL-4 (a) and stained for analysis by flow cytometry. Representative plot (b) showing gating of large CD14+ cells which were analysed for expression of CD11c (c), CD40 (f), CD86 (i) and CD83 (l) with positive stained cells (grey filled) and unstained cells (unfilled). The percentages of iMoDCs positive for CD11c (d), CD40 (g), CD86 (j) and CD83 (m) and surface expression levels shown as MFIs of CD11c (e), CD40 (h), CD86 (k) and CD83 (n) in individual mesothelioma patients (n = 46) and age-matched controls (n = 27) is shown. *p < 0.05.
Figure 3: iMoDCs from mesothelioma patients have a reduced capacity to process antigen

Immature MoDCs from mesothelioma patients and age matched controls were incubated for 1 hour with DQ-Ovalbumin. Representative dot plot (a) showing gating of MoDCs based on size and granularity. The capacity to process antigen was determined by emission of a signal in the FITC channel (b) and measured by flow cytometry; grey histogram represents cells incubated with DQ-OVA, white histogram represents control cells that did not receive DQ-OVA. Pooled data (c) of the mean fluorescent intensity (MFI) indicating relative antigen processing capacity of mesothelioma (n = 42) versus age matched (n = 29) MoDCs is shown as mean ± SE. **p < 0.005.
Figure 4: MoDCs from mesothelioma patients do not fully up-regulate CD83, CD40 and CD86 in response to maturation stimuli

Immature MoDCs generated from mesothelioma patients and age matched volunteers were stimulated with LPS (a) and cell surface molecules analysed by flow cytometry. Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured and shown as MFI of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in mesothelioma patients (n = 46) versus age matched (n = 27) iMoDCs. Pooled data is shown as mean ± SE.
Figure 5: LPS-matured MoDCs from mesothelioma patients lose their capacity to process antigen

Immature and LPS +/- IFNγ or CD40L activated MoDCs from mesothelioma patients and healthy age-matched volunteers were incubated for 1 hour with FITC-DQ-OVA. Pooled data of the percentage of DCs still processing antigen and MFIs indicating relative antigen processing capacity is shown as mean ± SE from age-matched volunteers (n = 29) and mesothelioma patients (n = 42). *p < 0.05.
Figure 6: LPS-matured DCs mesothelioma induce T cell proliferation
Immature and LPS +/- IFNγ or CD40L activated MoDCs were co-cultured with allogeneic CFSE-labelled lymphocytes for 7 days. Cells were collected, stained for CD4 and CD8 expression and analysed by flow cytometry. Lymphocytes were gated by size and CD4+ or CD8+ expression and the percentage of proliferating cells of total cells determined. Pooled data from healthy age-matched (n = 19) versus mesothelioma patients (n = 23) MoDCs is shown as mean ± SE.
Figure 7: MoDCs from mesothelioma patients secrete the same or higher levels of cytokines in response to stimulation

Culture media from LPS+-/- IFNγ or CD40L-stimulated MoDCs from mesothelioma patients and healthy age-matched controls were analysed by CBA for cytokine production. Pooled data for IL-10 (a), VEGF (b), TNF (c), IL12p70 (d) and IFNγ (e) secreted by MoDCs from mesothelioma patients (n = 45) and age-matched controls (n = 14) is shown as mean ± SE.
Figure 8: Increased survival correlates with circulating mDC1s and MoDCs that respond appropriately to maturational stimuli

Whole blood from mesothelioma patients was analysed for blood DC subpopulations and the number of circulating MDC1s plotted against survival from time of blood collection (a). The percentage of mesothelioma patient-derived LPS/IFNγ stimulated MoDCs able to process antigen measured using the DQ-OVA assay was plotted against survival (b). The percentage of cells expressing CD80 following stimulation with CD40L was plotted against survival (c). Similarly, CD80 expression levels (MFI) following stimulation with IFNγ were plotted against survival (d). All p values were determined using the Logrank Test.