Monocyte derived dendritic cells have reduced expression of co-stimulatory molecules but are able to stimulate autologous T-cells in patients with MDS

INTRODUCTION: Research has implied that the immune system plays a role in the pathogenesis of MDS and that T-cells are reacting to tumour antigen present on the surface of the malignant cells. This could imply that the immune system could be utilized to generate immune based therapy. The aim of this pilot study was to examine the feasibility of studying this further by analysing the interaction of dendritic cells with T-cells in a small cohort of MDS patients.

METHODS: Dendritic cells were generated in 6 MDS patients and 9 controls by culturing monocytes with GM-CSF and IL-4. After activation with LPS and TNFα, the dendritic cells were analyzed for expression of co-stimulatory and activation antigens. Thereafter, they were co-cultured with T-cells and the T-cell response was examined by measuring the % change in expression of the activation antigen CD69.

RESULTS: MDS MoDC had reduced expression of HLA-DR (p=0.006), CD11c (p=0.0004), CD80 (p=0.03) and CD86 (p=0.003), while resting T-cells from MDS patients had higher expression of the activation antigen CD69 on all subsets. The % change in CD69 expression increased significantly for both the control and MDS T-cells after co-culture with allogeneic dendritic cells, however this change was lower in the MDS group. Despite the increased CD69 expression prior to culture, MDS MoDC significantly up-regulated CD69 expression on autologous T-cells to values that were statistically higher than control cells.

CONCLUSION: This initial study suggests that the T-cells in MDS are able to respond to dendritic cells and are therefore probably not part of the malignant clone. It further implies that the dendritic cell population could be capable of presenting antigen and initiating an immune response and therefore further study is both feasible and warranted.

The myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders characterised by peripheral blood cytopenias, ineffective hematopoiesis and dysplasia of one or more bone marrow cell lines.1,2 It has been suggested that the immune system is activated in MDS patients and plays a role in the pathogenesis of the disease. This theory has been supported by studies which have demonstrated increased apoptosis in the bone marrow, oligoclonality of the T-cell population and increased expression of activation antigens on the surface of the T-cells.3-4 It has been hypothesised that these activated T-cell clones are reacting to antigens expressed on the surface of the malignant cells.5,6

Myeloid dendritic cells are effective antigen presenting cells and central to the immune reaction. After
encountering the antigen they mature, migrate through the tissues to secondary lymphoid organs where the processed antigen is presented to T-cells in association with Major Histocompatibility Complex MHC molecules. The T-cells become activated, proliferate and initiate an antigen specific immune response. However, antigen presentation by dendritic cells can also induce anergy which can result in the expansion of regulatory T-cells (Tregs) and the possible down-regulation of the immune response. This occurs if the dendritic cells do not mature fully or fail to express co-stimulatory molecules which T-cells require to become activated. Macrophages, monocytes and dendritic cells have been shown to be part of the MDS clone and previous studies have suggested that Monocyte derived Dendritic Cells MoDC cells in MDS patients are reduced in number and have a decreased ability to express activation antigens, secrete cytokines and stimulate allogeneic T-cells. These abnormalities could further lead to defective interaction with autologous T-cells and may also alter haematopoiesis.

Dendritic cells have been used in the treatment of many solid and haematological malignancies and some studies have been successful in generating immune responses. Recently, leukaemia-derived dendritic cells have been generated from patients with acute myeloid leukaemia and have been able to initiate anti-leukaemia T-cell responses in some cases. These studies suggest that dendritic cells and T-cells could be utilised to develop therapies that harness the patient’s own immune response to treat haematological malignancy. Therefore, in order to better understand the role of the immune system in myelodysplasia and to ascertain whether it would be feasible to further investigate MDS dendritic cells and induce them to present tumour antigen to T-cells, we performed a pilot study on a small cohort of patients. The aim was to examine the interaction of mature monocyte derived dendritic cells with both allogeneic and, more importantly, autologous T-cells in patients with symptomatic MDS.

METHODOLOGY

Patients and controls
The ability of dendritic cells to up-regulate co-stimulatory antigens and to activate autologous and allogeneic T-cells ex vivo was analysed in 6 patients and compared to 9 normal controls. The study was approved by the University of Cape Town Ethics Committee and both patients and normal control groups were required to read and sign a consent form prior to any samples being taken. The diagnosis of myelodysplasia was made according to criteria proposed by the World Health Organisation (WHO) classification of malignancies.

Isolation of monocytes and generation of immature monocyte derived dendritic cells
Monocytic cells are known to adhere to plastic. Therefore, in order to isolate the monocytes and generate dendritic cells the method of plastic adherence was utilised. 30 ml of peripheral blood was collected aseptically into sterile heparin vacutainer tubes and the mononuclear cells were separated from the whole blood using Ficoll density centrifugation (1.077 g/ml; Histopaque – Sigma–Aldrich). Thereafter, the cells were washed using RPMI (RPMI-1640, Sigma–Aldrich) and re-suspended in 24 ml RPMI containing 20% AB serum (Western Province Blood Transfusion Service; Cape Town, South Africa). 3 ml of the cell suspension with a median cell concentration of $1.33 \times 10^9$/ml ($r = 0.9–2.8$) in the controls and $0.9 \times 10^9$/ml ($r = 0.5–3.4$) in the MDS group, was added to each of the eight culture plates in order for plastic adherence to take place. After 2 h incubation at 37°C the non-adherent cells were removed, centrifuged and reconstituted with 1 ml RPMI. The % of non-adherent cells expressing the monocytic antigen CD14 and the T-cell antigen CD3 was analysed for quality control purposes. These cells were then discarded.

The generation of immature dendritic cells was achieved by adding 3 ml of RPMI which contained 20% AB serum, 10 ng/ml (1 $\times 10^4$ units per 1 $\mu$g) GM-CSF (G 5035, Sigma Aldrich) and 10 ng/ml IL-4 (1 $\times 10^4$ units per 1 $\mu$g) (I 4269, Sigma Aldrich) to the remaining cells the method of plastic adherence was utilised. The cells were incubated for 5 days at 37°C in a humidified CO$_2$ incubator and thereafter removed and analysed for the expression of dendritic cell antigens. The percentage of dendritic cell yield was calculated by dividing the cell count after culture with cytokines, with the cell number originally put into culture. The panel of antibodies used for analytical purposes consisted of HLA-DR (Becton Dickinson), CD11c (Becton Dickinson), CD80 (Immunotech), CD86 (Becton Dickinson), CD83 (Immunotech) and CD1a (Becton Dickinson). CD14 (Immunotech) and CD3 (Immunotech) were also included in the panel to establish the number of monocytes and T-cells present in the cell suspension. Analysis of the MoDC was performed using a Becton Dickinson FacsCalibur flow cytometer utilising a standard four colour protocol. Antigen presenting cells were identified as large cells expressing high forward and side scatter and the percentage positivity was compared to an isotypic negative control.
Stimulation of autologous T-cells in patients

Activation of dendritic cells
The ex vivo generated immature dendritic cells were counted using a particle counter (Model: ZF, Coulter Electronics – Hialeah, Florida, USA) and thereafter re-plated with a median cell concentration of $0.1 \times 10^6$/ml ($r = 0.05–0.24$) in the MDS patients and $0.16 \times 10^6$/ml ($r = 0.08–0.26$) in the control group. In order to mature and activate the dendritic cells, 10 ng/ml ($1 \times 10^5$ units per 1 µg) TNFα, Sigma Aldrich and 1 µg lipopolysaccharide (LPS) ($5 \times 10^5$ units per mg) (L6529, Sigma Aldrich) were added to the dendritic cell medium and incubated for a further 72 h at 37 °C in 5% CO₂. An aliquot of the activated dendritic cells was then analysed for changes in the expression of co-stimulatory and activation antigens using the same gating strategy, flow cytometer settings and panel of antibodies were employed for the analysis of the immature dendritic cells. The results were compared to a series of age matched normal controls and to the antigenic expression of the cells prior to activation and maturation.

Addition of T-cells
The effects of the activated MoDC on T-cells was analysed by co-culturing allogeneic or autologous lymphocytes with the mature dendritic cells. This was achieved as follows: 30 ml of peripheral blood was again venisected (7 days later) from each patient (autologous) and from third party allogeneic normal donors. After Ficoll separation, the cells were washed and enumerated. The autologous or third party allogeneic lymphocytes were then plated with the activated dendritic cells at a ratio of 1 dendritic cell to 10 lymphocytes in dendritic cell medium containing GM-CSF and IL-4. In order to determine the occurrence of an allogeneic response between residual autologous T-cells and donor T-cells, four of the plates containing MoDC (stimulator cells) were irradiated (30 Gy) prior to the addition of the donor T-cells, while the other four were processed without irradiation. The effects of the irradiation on the T-cells and dendritic cells were then compared. Normal control dendritic cells were also cultured in the same manner with autologous (their own) and with allogeneic lymphocytes (from another third party normal individual and the MDS patient). All the culture plates were incubated at 37 °C with 5% CO₂ for 72 h.

Harvest and analysis of T-cell response
After 72 h, both the non-adherent and adherent cells were removed from the culture plates using a mixture of phosphate buffered saline and EDTA. Two millili-
showed wide variation with a median of 5.7% (r = 2–43) compared to 6.7% (r = 4.1–20.5) in the normal samples. Flow cytometry was performed on the immature and on the mature dendritic cells after the activation step. Prior to activation, the percentage of immature MDS dendritic cells expressing the surface antigens HLA-DR (p = 0.06) and CD11c (p = 0.05) was reduced in comparison to the control group (Fig. 1).

Table 1. Patient demographics.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>IPSS score</th>
<th>Cytogenetics</th>
<th>% Blast in bm</th>
<th>Leucocytes (× 10⁹/l)</th>
<th>Platelets (× 10⁹/l)</th>
<th>Haemoglobin (g/dl)</th>
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<td>1</td>
<td>F</td>
<td>56</td>
<td>RCMD</td>
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<td>46XX</td>
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<td>70</td>
<td>RAEB</td>
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<tr>
<td>5</td>
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<td>RAEB</td>
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<td>2.57</td>
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<td>46XX</td>
<td>&lt;5</td>
<td>1.41</td>
<td>15</td>
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After activation, the dendritic cells from the MDS patients had significantly reduced expression of HLA-DR ($p = 0.006$) and CD11c ($p = 0.0004$). In addition, they failed to up-regulate the co-stimulatory molecules CD80 ($p = 0.03$) and CD86 ($p = 0.003$) which were significantly reduced when compared to normal controls (Figure 2B). However, the percentage of cells expressing the dendritic cell specific antigen CD83 was not significantly different to the control group ($p = 0.4$), implying that the number of dendritic cells was not decreased. Cells from the MDS group showed a wide variation in antigen expression as measured by the mean fluorescent intensity (MFI), which was reduced for HLA-DR, CD11c ($p = 0.01$), CD83 ($p = 0.06$), CD80 ($p = 0.03$), and CD86 ($p = 0.003$). These results indicate that after exposure to LPS and TNFα as stimulation agents, although the percentage yields and number of MoDC are similar, fewer cells express activation markers suggesting an abnormal development.

The response of T-cells after co-culture with dendritic cells (Figure 3A–C)

Comparative studies of lymphocytes cultured with irradiated and non-irradiated allogeneic dendritic cells demonstrated that there was no significant difference in the CD69 expression on CD3+, CD4+ and CD8+ T-lymphocytes. Due to limited cell numbers, it was therefore decided to discontinue further irradiation of the culture plates.

Subsequently, the interaction of the mature activated dendritic cells with both autologous and allogeneic T-cells was investigated. After 72 h, the response rate of the CD4, CD8 and CD3+ T-cell subsets was analysed by comparing the expression of the activation antigen CD69 both before and after incubation with the dendritic cells (Figure 1). The percentage of resting MDS T-cells expressing CD69 was higher than normal controls in the CD3+ ($p = 0.009$), CD4+ ($p = 0.05$) and CD8+ ($p = 0.05$) populations, suggesting that T cells from MDS patients were already activated (Figure 3A).

As expected, normal control T-cells co-cultured with normal allogeneic MoDC led to significantly increased expression of CD69 on all T-cell subsets. In contrast, normal T-cells cultured with dendritic cells from MDS patients showed very little change in CD69 expression compared to resting T-cells (Figure 3B). The median % change of CD69 expression before and after co-culture was significantly higher in the control group (T-cells cultured with normal dendritic cells) for CD3+ (1788 vs. 196; $p = 0.007$) and CD8+ (1736 vs. 300; $p = 0.006$) T-cell subsets.

The next set of experiments examined the response of T-cells after co-culture with their own monocyte derived dendritic cells (autologous response). T-cells from normal individuals co-cultured with their own generated mature MoDC showed a modest but significant increment in CD69 expression on the CD3+ ($p = 0.01$), CD4+ ($p = 0.07$) and CD8+ ($p = 0.04$) lymphocytes. In contrast, after co-culture with their own MoDC, the percentage of MDS T-cells expressing CD69 also increased significantly from baseline values ($p = 0.01$, 0.03 and 0.04 respectively; Figure 3C) and appeared to significantly exceed the responses by the normal T-cells (CD3 $p = 0.01$; CD4...
However, despite this brisk increase in the expression of CD69, the % change in expression was similar for both groups in all T-cell populations studied (535% vs 552%; p = 0.08; 388% vs. 536%; p = 0.1 and 685% vs. 380%; p = 0.87).

In summary, resting T-cells from MDS samples had a higher expression of CD69 than control samples, implying that they were in an activated state and although both MDS and control dendritic cells induced a significant change in CD69 expression on allogeneic normal T-cells, the % CD69 expression was significantly higher when cultured with normal dendritic cells. Significantly, when autologous MDS T-cells were co-cultured with their own MDS MoDC, the % change in CD69 expression was enhanced and equal to that obtained in the control group, implying an adequate T-cell response.

**DISCUSSION**

Dendritic cells have been utilised in the treatment of many solid and haematological malignancies and although further research is required, most studies...
have been successful in generating immune responses. Some of the poor clinical responses recorded in diseases such as myeloma have been attributed to dysfunctional dendritic cells which were unable to stimulate T-cells. Therefore, in order for immunotherapy to be successful, the ability of the dendritic cells to activate T-cells needs to be assessed. Recently, leukaemia-derived dendritic cells have been generated from patients with acute myeloid leukaemia and were able to initiate anti-leukaemia T-cell responses in some cases. Studies in our laboratory and by other investigators have implied that the excessive apoptosis and inhibition of colony growth described in MDS patients is a consequence not solely of the T-cells reacting with the CD34+ progenitor cells, but of the complex interaction of the malignant progeny with the bone marrow environment, including stromal cells, dendritic cells and other cells of the immune system.

In order to investigate the feasibility of studying this concept further, we performed a pilot study on six MDS patients in order to examine the role of T-cells and their complex relationship with antigen-presenting cells. Dendritic cells are heterogeneous, consisting of both myeloid and lymphoid subsets which follow different maturation pathways and produce subtypes which have distinct morphology, antigen expression and function. Myeloid dendritic cells are able to stimulate an inflammatory T-cell response which is capable of targeting tumour cells and because both monocytes and dendritic cells have been shown to be part of the MDS clone, the focus of our study was on this subtype of the dendritic cell.

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Previous studies have suggested that MoDC cells in MDS patients are reduced in number and have a decreased ability to up-regulate activation antigens and secrete cytokines. As a defective response to TNFα as well as poor ability to stimulate allogeneic T-cells has been reported, our study was aimed, in addition, to investigate the interaction of MoDC with autologous T-cells from MDS patients. Contrary to previous studies, the median % yield of immature MoDC in the MDS group was not significantly different from normal. This was further supported by the percentage of cells expressing the dendritic cell specific antigen CD83 which was similar to controls. In agreement with previous studies however, we observed that after stimulation with LPS and TNFα there was an adequate up-regulation of co-stimulatory antigens (CD80 and CD86), HLA-DR and CD11c in the MDS samples (Figure 2B). The decreased expression of CD80 (B7.1) and CD86 (B7.2) is an important finding as these antigens bind to CD28, CD40-ligand and other molecules expressed on the surface of T-cells, which are essential for their activation to take place. Decreased expression of CD80 and CD86 suggests that the T-lymphocytes may not be adequately stimulated, an observation associated with anergy and the possible proliferation of T-regulatory cells (Tregs) which could suppress the immune response. A study of peripheral blood Tregs in 52 MDS patients demonstrated an expansion of CD4+ Tregs in late disease and lower numbers in the earlier clinical subtypes. While we did not study Tregs generation, and our sample group is small, we observed that the activated MoDC in a patient with early stage disease retained the capacity to increase the expression of CD80 and CD86 and was able to stimulate the effective expression of CD69 on allogeneic T-cells. This was in contrast to patients who were in more advanced stages of MDS. This may be important as activation of the immune system may vary with disease progression.

In a further step, we examined the ability of allogeneic and autologous T-cells to up-regulate CD69 expression after co-culture with MoDC. We have also shown that when compared to control samples, the autologous CD3+, CD4+ and CD8+ T-cells have increased expression of CD69 even before co-culture with MoDC. In support of previous reports, our results demonstrated that in MDS, ex vivo generated dendritic cells have the capacity to stimulate normal allogeneic T-cells, however this was significantly reduced when compared to normal MoDCs (Figure 3B).

In addition, our study has revealed a novel and interesting observation in that the expression of CD69 on autologous MDS T-cells after co-culture with MoDC (Figure 3C) exceeded the allogeneic reaction. This enhanced expression of CD69 on autologous CD3+, CD4+ and CD8+ subsets led to an increased % change in the expression of CD69 that was equal to the normal T-cells co-cultured with their own MoDC. This could imply an anti-tumour T-cell response to the haemopoietic precursors which has been previously proposed. Therefore, although MoDC, derived from MDS patients may not have a similar phenotype to normal, they appear to be able to interact with autologous T-cells and could influence the immune response. It also implies that MDS T-cells are able to respond to dendritic cell stimulation and are probably not part of the malignant clone.
original research report

Multiple blood transfusions have been shown to stimulate immune cells with the consequent activation of T-cells. This could be important as our hospital is a referral centre and these MDS patients were already transfusion dependent upon entering the study. This could have contributed to the activated immune environment observed and could also have an influence on all studies which examine immune responses in MDS patients. Nevertheless, even though the expression of CD69 on MDS T-cells was increased pre-culture, in our cohort of patients, this expression increased even more post-culture with MoDC (Figure 3C).

The % change of CD69 expression on CD4+ T-cells could be relevant as this subset of cells consists of numerous sub-populations, all of which influence the immune response in different ways. These include both suppression by T regulatory cells (regs) and/or the induction of an inflammatory response (Th1).

Tregs cells include CD4+CD25+Foxp3+Tregs which are either naturally occurring or stimulated by tolerogenic dendritic cells, Tr1 cells which express the immunosuppressive cytokine IL-10 and Th3 Tregs which express TGF-β and lack CD25. A fourth subtype of the Treg cell has also been identified. This sub-group is CD4+, CD25−, Foxp3−, CD122+ and interestingly CD69+. They have high levels of TGF-1β which inhibits T-cell proliferation and experiments in cancer bearing mice have shown that as their tumours progress the numbers of CD4+CD69+CD25-Tregs increase, suggesting that this sub-type of Tregs could play an important role in the progression of malignancies. Our results have demonstrated an increase in CD4+CD69+ T-cells after co-culture with dendritic cells which could imply that the MDS dendritic cells may be stimulating a population of Treg cells which have the ability to inhibit an anti-tumour response. On the other hand, it has also been shown that there is an increase of Th1 cells in MDS and that IL-17 producing CD4+ cells are increased in the early stages of the disease.

CD4+ T-helper cells are also important in the maintenance of cytotoxic CD8+ T-cell activation and therefore could play a vital role in stimulating and maintaining an inflammatory response. Our initial results therefore indicate that future studies should focus on the influence of dendritic cells on all the subtypes of CD4+ T-cells. This should include the analysis of CD69+ Tregs and the expression of TGF-β.

We have also shown that the CD8+ T-cells had significantly increased CD69 expression prior and after co-culture (Figure 3A). In a study of trisomy 8+ MDS patients, all had significantly expanded oligoclonal CD8+ T-cells which inhibited clonogenic cell growth when in direct cell contact with haemopoietic progenitors. These findings implied that the CD8+ T-cells were responding to the presentation of antigens in association with MHC-Class I molecules and while the mechanism of this response was not clear this could be important in the generation of immune based therapies. The ability of the MDS dendritic cells to interact with target T-cells should therefore be further studied by stimulating them with specific antigens and thereafter measuring specific T-cell responses using MHCTetramers or other methods. In patients responsive to immunosuppressive therapy, significant T-cell stimulation was demonstrated against the Wilms tumour antigen (WT1) suggesting that the WT1 protein could be one of the antigens which initiate an immune response in MDS.

The WT1 antigen could however also stimulate the production of WT1 specific Treg cells which recognise the WT1 antigen. Experiments by Lehe et al. generated anti WT1 specific T-cell lines which displayed a Th2 phenotype, recognised HLA-matched WT1+ leukemic cells and inhibited the activity of allogeneic T-cells without any direct cell contact. These WT1 specific Treg cells were able to induce apoptosis of the dendritic cells but although they inhibited the proliferation of CD8+ cytotoxic T-cells, they did not have an effect on their cytotoxic function. These results are important as the generation of antigen specific T-cells in MDS and other malignancies may be limited by the proliferation of antigen specific Tregs which may require depletion in order for success to be achieved.

The results of this initial study suggest that the mechanism of dendritic cell interaction with T-cells is preserved and that T-cells are probably normal and not part of the malignant clone. Therefore, the abnormal activated environment that is responsible for many of the features observed in patients with MDS is probably the result of the complex interaction of all the cells in the bone marrow including the stem cells themselves, the bone marrow stroma and the abnormal cytokine milieu. The ability of the T-cells to respond to the MoDC could also suggest that the immune system may be utilised in the development of immune based therapies. However, as previously described, dendritic cells in malignant environments have the ability to not only stimulate an inflammatory response but also the proliferation of Tregs. Although CD69 has been well described as an early activation molecule, recent evidence suggests that this antigen could also be expressed by Tregs and plays a role in inhibiting T-cell proliferation. Therefore, although we have demonstrated that MDS dendritic cells are able to stimulate CD69 expression on T-cells, further analysis of these

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CD69+ T-cell populations and their relationship with the dendritic cells in MDS is required before successful immune based therapies can be achieved.

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