Alveolar macrophages from systemic sclerosis patients: evidence for IL-4-mediated phenotype changes

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Alveolar macrophages from systemic sclerosis patients: evidence for IL-4-mediated phenotype changes. *Am J Physiol Lung Cell Mol Physiol* 286: L1202–L1209, 2004. First published January 16, 2004; 10.1152/ajplung.00351.2003.—The mechanism of chronic lung inflammation leading to lung fibrosis is unknown and does not have a characteristic inflammatory macrophage phenotype. This study was undertaken to determine whether a change in macrophage phenotype could account for chronic lung inflammation. In this study, human alveolar macrophages (AM) from subjects with systemic sclerosis (SSc) were obtained from bronchoalveolar lavage (BAL) and characterized on the basis of function (response to LPS), phenotype, and relative cell-surface B7 expression. AM from the subjects’ disease-involved and noninvolved lung lobes were compared with each other and to AM from normal volunteer BAL. AM from involved SSc lobes produced significantly more interleukin (IL)-1β and PGE2 than AM from uninvolved lobes in response to LPS, but there was no spontaneous production of either mediator. The activator AM phenotype designated by RFD1+ surface epitope was significantly elevated in SSc BAL samples compared with normal BAL, although there were no differences comparing involved vs. noninvolved lobes within SSc subjects. The major histocompatibility complex II costimulatory molecule B7.2 was also significantly elevated in SSc AM compared with normal AM, again with no differences between involved and noninvolved lobes. In an attempt to determine environmental influences on AM phenotypes, normal AM were cultured in vitro with IFN-γ, IL-3, IL-4, IL-10, IL-12, or dexamethasone for 6 days. Of the cytokines examined, only IL-4 induced significant increases in both the activator phenotype RFD1+ and B7.2 expression. Taken together, these results indicate that IL-4 could account for proinflammatory AM phenotype changes and B7 surface-marker shifts, as seen in subjects with SSc.

RFD1; RFD7; B7.2; macrophage subpopulations

SYSTEMIC SCLEROSIS (SSc) or scleroderma is a complex systemic disorder characterized by uncontrolled deposition of collagen and other matrix proteins in the connective tissue of the skin, internal organs, and vasculature (20). The initiation of the disease may involve genetic predisposition, immune dysfunction, and environmental exposure factors, but the exact cause of SSc remains unknown (33). Although the primary internal organs affected by SSc are in the gastrointestinal tract (37), the lungs are the second most affected internal organ system in SSc featuring the occurrence of pulmonary hypertension and pulmonary fibrosis (37). Lung fibrosis develops as a result of chronic alveolitis initiating collagen deposition from proliferating fibroblasts, which leads to reduced respiratory volume and impaired gas diffusion (36). However, the mechanism(s) of chronic alveolitis remains unknown.

Several cytokines and growth factors have been implicated in the SSc disease process. Interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, transforming growth factor-β, PDGF, TNF-α, IFN-γ, and soluble IL-2 receptor are elevated in the blood of SSc patients at various stages of the disease development (6, 10, 14, 18, 19, 28). Some of these cytokines could simply be the result of inflammation (IL-1, IL-8, TNF-α). IFN-γ may be an antagonist of the disease process, as it inhibits fibroblast proliferation and collagen production and may be a counterregulatory response to SSc (24). However, some cytokines, such as IL-2, soluble IL-2r, IL-4, IL-5, IL-6, IL-13, and transforming growth factor-β are considered to be primary mediators of the disease process (15). IL-4 has been shown to stimulate collagen production in SSc and normal fibroblasts in vitro culture experiments (12). In addition, studies are focusing on alternative splice variants of IL-4 (found in SSc patients) as a unique initiator of disease (3). Both wild-type and alternative splice variant IL-4 mRNA have been found in the CD8+ bronchoalveolar lavage (BAL) cells from SSc patients, and it correlates positively with lung function decline (4).

Changes in helper and inducer T lymphocyte (Lymph), suppressor and cytotoxic T Lymph, and natural killer cell phenotypes, consistent with those described in SSc lung fibrosis, are also found in other autoimmune diseases, such as Sjögren’s syndrome, characterized by the absence of lung fibrosis (13). This suggests that human alveolar macrophage (AM) activation may be a central driving feature in the SSc pulmonary fibrotic process (13). Functional AM phenotypes have been described on the basis of RFD1 and RFD7 surface epitopes (35). The activator AM are RFD1+ and are described as a strong stimulator of T Lymphs, weakly phagocytic, dendritic like, and nonadherent to glass (35). The suppressor AM are RFD1+ and RFD7+ and are described as poor stimulators of T Lymphs, phagocytic, adherent, and may actively suppress the activity of RFD1+ cells by an undefined mechanism (35). AM phenotype ratios can be altered or shifted to a more activator status by environmental factors in vitro (16, 17) and may be under the influence of inflammatory mediators concomitant with disease (38). In addition, alterations in the ratio of activator AM to suppressor AM are consistent with several inflammatory lung disorders (21, 29, 39). Therefore, changes in AM phenotype could be initiated in SSc-associated lung fibrosis.
The major histocompatibility complex II costimulatory molecule B7.2 on AM could also play a significant role in SSc disease progression. Antigen-presenting cells, such as AM, require a second costimulatory signal from either B7.1 or B7.2 surface molecules to optimally activate T cells (41). Although controversial, several studies have determined that elevated B7.2 expression in AM is associated with Th2 immune pathway activation (23, 40, 9). This Th2 priming effect has also been established for B cells that upregulate B7.2 (31), but not dendritic cells in which B7.2 overexpression appears to favor the Th1 pathway (23). Th2-dominated cellular responses have been reported to play a role in the pathogenesis of progressive SSc (30), especially with regard to cryptogenic fibrosing alveolitis (22).

The purpose of this study was to examine the functional status and phenotype of AM in affected and unaffected lung lobes of SSc patients and compare these observations to normal AM. The overall hypothesis is that SSc patients produce excess wild-type and/or splice-variant IL-4 in the lung, which initiates changes in AM phenotype and B7 expression before objective disease involvement, resulting in sustained inflammation and Th2 immune response. Furthermore, these changes precede general lung inflammation.

**METHODS**

**Criteria for SSc subjects.** The following procedures were approved by the University of Texas, Houston Health Science Center’s Committee for the Protection of Human Subjects Internal Review Board. All subjects used in this study signed “informed consent” documents. Ten female subjects meeting the American Rheumatism Association diagnostic criteria (1) for the diagnosis of SSc, referred for pulmonary medical evaluation and BAL for clinical treatment, were used in this study. The descriptive data for each subject can be found in Table 1. The duration of disease was variable from 4 mo to 5 yr (means ± SD = 2.083 ± 1.66 yr). The age of the subjects ranged from 23 to 65 yr (means ± SD = 43.5 ± 16.55 yr). These data were normally distributed by Kolmogorov-Smirnov test for normality. The ethnic background of the 10 subjects was diverse. The severity of SSc and presence of secondary clinical problems were variable among subjects, as was the use of prescription drugs.

All subjects underwent complete pulmonary function testing on Medgraphics 1085/D (Medical Graphics, St. Paul, MN) equipment. Lung volumes consisting of vital capacity, residual volume, and total lung capacity were performed by a computerized analyzer, with the

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age, yr</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>SSc Duration</th>
<th>Medications</th>
<th>Lobes Lavaged (HRCT Scoring)</th>
<th>Clinical Characteristics (All Subjects With Diffuse SSc Except Subject 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>Female</td>
<td>Black</td>
<td>6 mo</td>
<td>None</td>
<td>RUL(1)*</td>
<td>No pulmonary complaints</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>Female</td>
<td>White</td>
<td>5 yr</td>
<td>Diltiazem, ASA, Furosemide</td>
<td>RUL(0)* RLL(1)* LLL(2)* LLL(3)*</td>
<td>Severe SOB, Esophageal disease, Severe pulmonary HTN</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>Female</td>
<td>Asian</td>
<td>2 yr</td>
<td>None</td>
<td>RUL(0) RLL(1)*</td>
<td>Severe SOB, Hypoxemic with pulmonary HTN, Raynauld’s Arthralgias</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>Female</td>
<td>White</td>
<td>5 yr</td>
<td>Prednisone, D-Penicillamine, Warfarin, Captopril, Sulindac</td>
<td>RUL(1)* RLL(1)* LLL(4)*</td>
<td>Raynauld’s Hypoxemic with pulmonary HTN</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>Female</td>
<td>Black</td>
<td>4 mo</td>
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<td>No pulmonary complaints</td>
</tr>
<tr>
<td>6</td>
<td>46</td>
<td>Female</td>
<td>White</td>
<td>1 yr</td>
<td>None</td>
<td>RUL(0) RLL(2)* LLL(2)*</td>
<td>Occasional SOB with exertion</td>
</tr>
<tr>
<td>7</td>
<td>63</td>
<td>Female</td>
<td>Hispanic</td>
<td>2 yr</td>
<td>Nifedipine, ASA, Levothyroxine</td>
<td>LLL(2)*</td>
<td>Significant Raynauld’s Hypoxemic with pulmonary HTN</td>
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<tr>
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<td>56</td>
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<td>Hispanic</td>
<td>2 yr</td>
<td>Prednisone, Paroxetine, Enalapril, Omeprazole, D-penicillamine</td>
<td>RLL(1)*</td>
<td>Raynauld’s SOB with exertion</td>
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<tr>
<td>9</td>
<td>34</td>
<td>Female</td>
<td>Black</td>
<td>1 yr</td>
<td>Nifedipine</td>
<td>RUL(0) RML(0) LLL(2)* RLL(2)*</td>
<td>Esophageal disease, Hypoxemic with pulmonary HTN, Raynauld’s</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>Female</td>
<td>Black</td>
<td>2 yr</td>
<td>None</td>
<td>RML(0) LLL(0)</td>
<td>No pulmonary symptoms Limited SSc</td>
</tr>
</tbody>
</table>

SSc, systemic sclerosis; ASA, acetylsalicylic acid; SOB, shortness of breath; HTN, hypertension. The abbreviations in the lobe column are as follows: 1st letter: R, right; L, left; 2nd letter: U, upper, M, middle; L, lower, 3rd letter: L, lobe. *SSc lobe involvement.
subject sitting. The highest value for the sum of the forced expiratory volume at 1 s and forced vital capacity of three acceptable maneuvers was used. The diffusing capacity was measured as a single-breath diffusion capacity for carbon monoxide. Interpretations utilized normal subjects as per Crapo et al. (7) and were performed as recommended by the American Thoracic Society guidelines (2). All subjects revealed restrictive physiology as demonstrated by total lung capacity and forced vital capacity of 10% or less of predicted. Reductions in diffusing capacity for carbon monoxide of 15% or less of predicted normal subjects were present in all subjects. The pulmonary function data for the subjects can be found in Table 2.

Lung lobe involvement was assessed by high-resolution computed tomography (HRTC). A GE CTI series scanner with a scanning protocol utilizing prone positioning to minimize profusion artifact, with slice thickness of 1.5 mm every 10 mm of scanning and utilizing a “bone” reconstruction algorithm was used. The appearance of all scans was assessed by a single radiographer skilled in the interpretation of interstitial lung disease on HRTC. A grading system adapted by Muller et al. (25, 26) was utilized to assess each lobe as follows: 0, normal appearance; 1, parenchymal opacification (ground-glass opacity) alone; 2, parenchymal opacification more extensive than a reticular pattern; 3, parenchymal opacification equal in extent to reticular pattern; 4, reticular pattern more extensive than parenchymal opacification; and 5, reticular pattern alone. The scores for each subject’s lavaged lung lobe can be found in Table 1, with a higher value indicative of greater tissue changes.

Isolation of lung cells from SSc subjects. Specific lung lobes were lavaged with instillations of 80-ml sterile saline, which yielded ~50 ml of lavage fluid per lobe. This was kept on ice until the cell isolation. Specific details of the lung BAL procedure can be found elsewhere (32). One to four lung lavages were lavaged per subject, depending on accessibility and the subject’s tolerance for the procedure. Cells were isolated from the lavage fluid by centrifugation at 400 g (IEC centrifuge, Marietta, OH). The saline supernatant was aspirated and discarded, the cell pellet was resuspended in small-volume (1 ml) RPMI (Gibco BRL) with 10% heat-inactivated, human AB serum (ICN Biomedical) with antibiotics (50 U/ml penicillin, 50 µg/ml gentamicin, and 50 µg/ml streptomycin). Cell count was determined with the ZBI Coulter Counter. Lavages yielded an average of 20 × 10^6 cells that were >92% macrophages as identified by Hema 3 leukocyte kit staining. Cells were >90% viable by Trypan blue exclusion. Hema 3 staining was also used for all morphological examinations in these studies.

Cells (2 × 10^6) were plated on tissue culture-treated plastic six-well plates (Corning, Corning, NY), and the AM were allowed to adhere in a 2-h incubation at 37°C in a water-jacketed, 5% CO₂ incubator (Queue). The nonadherent cell fraction was then aspirated, the plates were washed once with PBS, and then media were added for the long-term culture of adherent AM. Experimental culture conditions were derived by the addition of either nothing [control (Cnt)], 200 U/ml IFN-γ, 0.5 µM dexamethasone (Dex; Sigma Chemical, St. Louis, MO), 10 ng/ml IL-4, 10 ng/ml IL-10, 1 ng/ml IL-3, or 10 ng/ml IL-12. Recombinant cytokines were from Biosource International (Camarillo, CA). Cytokine and Dex concentrations used in these studies were at saturation and selected based on pilot studies. For all cultures, the medium with cytokines was replaced every 2 days for 6 days. At the termination of the 6-day cell culture, the plates were scraped with a plastic policeman, and the cell suspension was centrifuged at 400 g and 25°C. The culture media were aspirated, and the cell pellet (1 × 10^6 cells) was resuspended in 500 µl of PBS with 3.5% BSA for cell surface-marker staining.

Table 2. Pulmonary function data for 10 SSc subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>TLC, liters</th>
<th>% TLC Predicted</th>
<th>FVC, liters</th>
<th>%FVC Predicted</th>
<th>DLCO, mmHg·l⁻¹</th>
<th>%DLCO Predicted</th>
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<td>1</td>
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<td>73</td>
<td>2.3</td>
<td>78</td>
<td>13.5</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
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<td>2.1</td>
<td>69</td>
<td>6.9</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>2.7</td>
<td>61</td>
<td>1.8</td>
<td>52</td>
<td>22.1</td>
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</tr>
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<td>3.8</td>
<td>76</td>
<td>2.3</td>
<td>76</td>
<td>4.9</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>3.2</td>
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<td>2.5</td>
<td>68</td>
<td>11.8</td>
<td>47</td>
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<td>6</td>
<td>3.3</td>
<td>68</td>
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<td>57</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
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<td>1.1</td>
<td>39</td>
<td>3.1</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>3.0</td>
<td>75</td>
<td>1.9</td>
<td>80</td>
<td>9.6</td>
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</tr>
<tr>
<td>9</td>
<td>2.7</td>
<td>66</td>
<td>1.6</td>
<td>56</td>
<td>7.9</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>2.6</td>
<td>61</td>
<td>1.5</td>
<td>46</td>
<td>11.2</td>
<td>42</td>
</tr>
<tr>
<td>Mean</td>
<td>3.0</td>
<td>65.2</td>
<td>1.9</td>
<td>63.4</td>
<td>10.7</td>
<td>41.5</td>
</tr>
<tr>
<td>SD</td>
<td>0.5</td>
<td>10.1</td>
<td>0.5</td>
<td>14.2</td>
<td>5.5</td>
<td>14.9</td>
</tr>
</tbody>
</table>

AM. Characterization of lung macrophages in Scleroderma. Specific lung lobes were lavaged with instillations of 80-ml sterile saline, which yielded ~50 ml of lavage fluid. The lavage fluid was kept at 4°C until cells were isolated by centrifugation at 400 g (IEC centrifuge). The saline supernatant was aspirated and discarded, and the cell pellet was resuspended in small-volume (1–5 ml) RPMI (Gibco BRL) with 10% heat-inactivated, human AB serum (ICN Biomedical) with antibiotics (50 U/ml penicillin, 50 µg/ml gentamicin, and 50 µg/ml streptomycin). Cell count was determined with the ZBI Coulter Counter. Lavages yielded an average of 20 × 10^6 cells that were >92% macrophages as identified by Hema 3 leukocyte kit staining. Cells were >90% viable by Trypan blue exclusion. Hema 3 staining was also used for all morphological examinations in these studies.

Isolation and long-term cultivating of normal human AMs. Cells for these experiments were obtained by BAL of normal, nonsmoking volunteers by methods previously described (32). Several lobes were lavaged by installations of 240–300 ml of sterile saline, which resulted in recoveries of 200–260 ml of lavage fluid. The lavage fluid was kept at 4°C until cells were isolated by centrifugation at 400 g (IEC centrifuge). The saline supernatant was aspirated and discarded, and the cell pellet was resuspended in small-volume (1–5 ml) RPMI (Gibco BRL) with 10% heat-inactivated, human AB serum (ICN Biomedical) with antibiotics (50 U/ml penicillin, 50 µg/ml gentamicin, and 50 µg/ml streptomycin). Cell count was determined with the ZBI Coulter Counter. Lavages yielded an average of 20 × 10^6 cells that were >92% macrophages as identified by Hema 3 leukocyte kit staining. Cells were >90% viable by Trypan blue exclusion. Hema 3 staining was also used for all morphological examinations in these studies.

Cells (2 × 10^6) were plated on tissue culture-treated plastic six-well plates (Corning, Corning, NY), and the AM were allowed to adhere in a 2-h incubation at 37°C in a water-jacketed, 5% CO₂ incubator (Queue). The nonadherent cell fraction was then aspirated, the plates were washed once with PBS, and then media were added for the long-term culture of adherent AM. Experimental culture conditions were derived by the addition of either nothing [control (Cnt)], 200 U/ml IFN-γ, 0.5 µM dexamethasone (Dex; Sigma Chemical, St. Louis, MO), 10 ng/ml IL-4, 10 ng/ml IL-10, 1 ng/ml IL-3, or 10 ng/ml IL-12. Recombinant cytokines were from Biosource International (Camarillo, CA). Cytokine and Dex concentrations used in these studies were at saturation and selected based on pilot studies. For all cultures, the medium with cytokines was replaced every 2 days for 6 days. At the termination of the 6-day cell culture, the plates were scraped with a plastic policeman, and the cell suspension was centrifuged at 400 g and 25°C. The culture media were aspirated, and the cell pellet (1 × 10^6 cells) was resuspended in 500 µl of PBS with 3.5% BSA for cell surface-marker staining.

AM phenotype immunostaining. The monoclonal antibodies to RFD1 (murine IgM) and RFD7 (murine IgG) surface antigens (Serotec; Kidington, Oxford, UK) were added concomitantly to the cell suspension at a 1:50 dilution (10^9 cells) was resuspended in 500 µl of 500 µl PBS with 3.5% BSA for cell surface-marker staining.

ELISA kits (Biosource International) were used to assay these mediators in the 24-h cell culture supernatants. Assays were performed according to the kit’s protocol. Each sample was measured in duplicate and averaged for a single value. The 96-well plates were analyzed for optical density by using a Spectra Max 340 (Molecular Dynamics), and the values were converted to picograms per milliliter by internal known concentration standards. All presented values are adjusted for AM cell differentials.
washed three times in PBS, suspended in 1% paraformaldehyde (PBS buffered), and stored at 4°C before flow cytometric analysis.

Flow cytometry. Flow cytometry was performed on a Coulter Epics Elite flow cytomter (Coulter, Miami, FL) by using the Elite software. With the use of forward and side scatter of the total cell populations, gates were drawn to include macrophages based on size and granularity of the cells. The instrument was calibrated with FITC- and PE-coated beads to compensate for any overlap within the green and orange/red fluorescence wavelengths. Cells stained without the inclusion of primary antibodies resulted in no significant staining. Cnt included unstained cells, cells that were stained with secondary antibodies only (negative Cnt), cells stained with Ig isotype Cnt (negative Cnt), and cells stained for only one surface marker (positive Cnt).

Statistical analysis. Flow cytometry results were expressed as percentage of cells stained with a given antibody or combination of antibodies. For 6-day AM cultures, phenotype expression was normalized to the day 0 baseline values (phenotype expression on the freshly isolated cells) and expressed as a fold increase or decrease. Day 6 Cnt values were compared with day 0 by Wilcoxon signed-rank sum test. All other analyses used in this study involved a one-way ANOVA followed by Dunnett’s multiple comparison to a single Cnt group or Bonferroni’s multiple comparison test for selected pairwise comparisons. Where there was no statistically significant difference between the affected and unaffected lobes of SSc subjects, the data were combined to produce more statistical power in the analysis.

RESULTS

BAL cell counts and differentials. Cells were obtained from normal subjects, in addition to the affected and unaffected lobes of SSc subjects determined from HRCT results, by BAL as described in METHODS. Figure 1 illustrates the cell-type differentials of these three populations. Cells obtained from the SSc unaffected lung lobes appear to have a normal cell distribution, as they are similar to the observed values in normal BAL. In contrast, the cell differentials from the SSc subjects’ affected lung lobes featured an influx of neutrophils (polymorphonuclear neutrophils) and/or eosinophils (EO), indicative of an inflammatory condition. The increase in EO, although not statistically significant, represented an average fourfold increase over normal values. There was an average threefold increase in polymorphonuclear neutrophils. These increases were highly variable from subject to subject and lobe to lobe. Some affected lobes featured exclusively neutrophilia or eosinophilia, and some had both. The cell counts (cells/ml BAL fluid) were not statistically different between these groups (data not shown), indicating that the number of AM was significantly decreased (~17%) in SSc-affected lung lobes. There was no change in the Lymph distribution between any of the groups.

Assessment of AM function following LPS stimulation. AM obtained from SSc subjects were cultured with and without LPS (1 µg/ml) for 24 h, as described in METHODS. Figure 2 shows the AM mediator release, comparing AM from the affected and unaffected lung lobes. Figure 2A illustrates the PGE2 levels produced by AM, and Fig. 2B shows the cytokine (IL-1β) levels produced by AM. All values were adjusted for the absolute number of AM. Unstimulated AM, regardless of lobe origin, did not spontaneously release either mediator. In contrast, LPS exposure initiated the release of both mediators. The AM values from unaffected lobes were not statistically different from normal AM values (data not shown). The AM from affected lobes produced significantly more PGE2 (twofold) and IL-1β (threefold) than AM from unaffected lobes.
This increased mediator release is consistent with the presence of activated or “primed” AM in the affected lobe’s proinflammatory environment. Taken together, these results are consistent with the existence of an inflammatory state in the affected lobes of the lung, but they do not explain what factor(s) may account for disease initiation in SSc lungs.

Changes in AM B7 expression and phenotype. AM from normal and SSc BAL were stained for B7 and RFD surface markers immediately after isolation. In contrast to the above results, there were no statistically significant differences in the B7 or RFD distributions in AM from affected and unaffected SSc lung lobes (data not shown), so these data were combined for comparison to normal AM. The B7 expression in AM (normal vs. SSc) is shown in Fig. 3A. Coexpression of B7.1 and B7.2 in AM was relatively low (5% of total AM) and was not statistically different between normal and SSc AM. AM expressing only B7.1 were extremely low (<0.5%) and were not included in this presentation. However, there was a statistically significant increase (40%) in SSc AM expressing only B7.2.

Relative RFD expression is shown in Fig. 3B. There was a statistically significant increase in SSc AM expressing only RFD1+ compared with normal AM. Thus there was a threefold increase in the number of activator AM. Although not statistically significant, there were decreases in the numbers of RFD7+ and coexpressing RFD1+ and RFD7+ AM from SSc subjects. There was a threelfold decrease in the mature AM phenotype (RFD7+) and a 20% decrease in the suppressor AM phenotype (RFD1+ and RFD7+). With these changes, the activator-to-suppressor AM ratio was altered in SSc subjects. The normal distribution of RFD1+ to RFD1+ and RFD7+ is 1:10. This ratio is 1:3 in AM from SSc subjects, regardless of lung lobe disease involvement.

Effect of cytokines and Dex on AM B7 expression in vitro. In these experiments, AM were cultured for 6 days with either IFN, Dex, IL-3, IL-4, IL-10, or IL-12 or were left untreated (Cnt), as described in METHODS and monitored for shifts in B7 expression. The B7 molecules were also measured on freshly isolated AM (preculture) to serve as a baseline for every culture. Due to large between-subject variance, the data were normalized and expressed as a “fold increase” in B7 surface marker expression over baseline (day 0). IL-3 and IL-12 had no effect on the B7 markers, and they were excluded from the analysis and data presentation. Cnt cultures showed no significant deviations from B7 expression on day 0. Cells expressing only B7.1 were extremely rare (<0.5%), and they were excluded from the analysis.

Figure 4A illustrates the relative changes in AM coexpressing B7.1 and B7.2 cultured for 6 days. There were no significant changes in B7 coexpression, although IFN did increase B7.1 and B7.2 coexpression by 80% over baseline AM values. In contrast, Fig. 4B shows a significant increase produced by IL-4 in AM expressing the B7.2 marker only. This represents an 80% increase over Cnt and baseline. This is significant when the absolute number of AM is taken into consideration. There were seven times more AM expressing B7.2 than B7.1 and B7.2 coexpressing AM. Dex and IL-10 appeared to downregulate the B7.2 marker slightly (~20%).

Effect of cytokines and Dex on AM phenotypes in vitro. In these experiments, AM were cultured for 6 days (preliminary studies showed no changes in RFD were evident after 1 day of culture, started to appear after 3 days of culture, and were optimal at 6 days) with IFN, Dex, IL-3, IL-4, IL-10, or IL-12 or were left untreated (Cnt), as described in METHODS and monitored for shifts in phenotype expression. The RFD markers were also measured on freshly isolated AM (preculture) to serve as a baseline for every culture. Due to large between-subject variance, the data were normalized and expressed as a fold increase in RFD surface marker expression over baseline (day 0). Similar to the B7 observations, IL-3 and IL-12 had no effect on the RFD markers, and they were excluded from the analysis and data presentation. Cnt cultures showed no significant deviations from RFD expression compared with day 0.

Figure 5A illustrates the effect of these cytokines and Dex on RFD1+ expression (activator phenotype). The only two cytokines that produced a statistically significant increase were IFN

![Image](https://www.jensenlindsey.org/Resources/3_1.png)
and IL-4. IFN produced a 10-fold increase in RFD1+ AM. This translated into 50% of the IFN cultured cells expressing RFD1 only. Similarly, IL-4 produced an eightfold increase in RFD1+ cells, which represented 40% of the total AM, on average. IL-10 produced a nonsignificant threefold increase in RFD1+, and Dex showed no significant deviation from day 0 values.

The effect of cytokines and Dex on the mature AM phenotype (RFD7+) is shown in Fig. 5B. Dex was the only factor that significantly enhanced RFD7+ expression in AM. IL-10 produced a doubling of RFD7+ cell over Cnt, but this was not significant. IL-10 appears to be a strong initiator of RFD7+ in differentiating monocytes (23) but apparently has limited influence in differentiated AM. IFN and IL-4 produced nonsignificant decreases in this phenotype (50 and 10%, respectively).

Figure 5C shows the effect of cytokines and Dex on RFD1+ and RFD7+ phenotype expression (suppressor phenotype). There was no statistically significant deviation from baseline values in any culture examined. Nevertheless, IFN and IL-4 produced 38 and 33% reduction in RFD1+ and RFD7+ cells, respectively. This, taken in the context that this was the largest normally occurring phenotype, indicated that even small percent changes in this phenotype represented a relatively large number of cells.

DISCUSSION

The parenchymal involvement of the lungs in SSc is characterized by subjective dyspnea, restrictive physiology, and reduced gas exchange. The role of the AM in this pathology is not clearly understood, although macrophages appear to be involved in many chronic lung inflammatory conditions. It is clear, however, from this study that the AM changed phenotypes (activation states) by a factor or factors before lobe inflammation and obvious radiological changes in the lung, in addition to being influenced by an inflammatory microenvironment.
SSc lung lobe involvement is traditionally characterized by neutrophilia and eosinophilia in the absence of increased Lymphs (34). In this study, there was no consistent quantifiable pattern regarding the increase of neutrophils and EO from involved lung lobes. Some subjects displayed either eosinophilia or neutrophilia, and some had a combination of both. However, there was no BAL sample from SSc-involved lung lobes that displayed a “normal” cell distribution.

The augmented mediator release in SSc involved lobe AM in response to LPS presented in Fig. 2 is consistent with known AM functional changes in an inflammatory lung environment (27). The primed AM response to LPS could be due to an increased expression of CD14 receptors, which have been found in the AM of SSc patients (35). This priming effect is associated with an increase in neutrophils and increased production of CXC chemokines (8), specifically cytokine-induced neutrophil chemoattractant protein (11). Changes in B7 and RFD surface markers in AM (Fig. 3, A and B, respectively), before and concomitant with lung inflammation, are a feature of SSc lung involvement. These changes in AM may be unrelated to inflammation or may be an initiator of inflammation, because the AM changes are apparent in SSc uninvolved lung lobes and continue after disease involvement. Elevated inflammation or may be an initiator of inflammation, because the AM changes are apparent in SSc uninvolved lung lobes and continue after disease involvement. Elevated

Elevated} B7.2 has been associated with enhanced Th2 antigen-presentation and prolongation of the Th2 cytokine response to LPS presented in Fig. 2 is consistent with known AM functional changes in an inflammatory lung environment. However, there was no BAL sample from SSc-involved lung lobes. Some subjects displayed either eosinophilia in the absence of increased Lymphs (34). In this study, there was no consistent quantification of the number of Lymphs in the SSc BAL (Fig. 1), but that does not negate the possibility that the functional phenotypes of these cells have shifted under the influence of other cytokines or growth factors. We were unable to detect IL-4 in the lavage fluid of the SSc subjects. However, the IL-4 concentration may have been below the limit of detection due to the dilution of lung lining fluid. Furthermore, the BAL fluid reflects only a fraction of the microenvironmental factors available to the cells. Therefore, tissue levels of IL-4 may be much more important in driving macrophage phenotype changes. Another possibility was that IL-4 initiated the process and was no longer present. There are several other cytokines reported to be elevated in SSc BAL fluid, which could potentially have effects on AM. TNF-α, IL-8, macrophage inflammatory protein-1α, and RANTES (regulated on activation, normal T-cell expressed, and presumably secreted) have all been elevated in SSc BAL fluid (5). TNF-α and IL-8 are associated with avoelitis and thus inflammation, whereas macrophage inflammatory protein-1α and RANTES were preinflammatory mediators (5). IL-5, also found elevated in the lung Lymphs of SSc patients (4), could be another possible mediator of these AM changes. IL-1β could be another possible influence in the inflammatory process. None of these tests was performed in our study, but they could provide additional avenues for investigation.

In conclusion, the results of this study suggest that AM in SSc patients are under the influence of pre- and postinflammatory mediators, and that changes in AM B7 expression and phenotype precede the inflammatory milieu. In turn, AM may be playing an active role in the disease process. Excess IL-4 (wild-type and/or splice variant) is a logical cause of these AM changes based on in vitro evidence and published observations. This would further suggest that SSc is at least partially driven by a Th2 process.

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REFERENCES


