Relationship between granulocyte activation, pulmonary granulocyte kinetics and alveolar permeability in extrapulmonary inflammatory disease

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1. The aim of the study was to examine the relationship between granulocyte activation, pulmonary intravascular granulocyte transit, pulmonary extravascular granulocyte migration and lung injury in patients with systemic conditions (bone marrow transplant recipients, inflammatory bowel disease and systemic vasculitis) in which abnormalities of pulmonary granulocyte traffic have previously been reported.

2. A double ^{111}In–^{99m}Tc granulocyte labelling technique was used for quantification of granulocyte kinetics in 23 patients, of whom five were control patients. The pulmonary vascular granulocyte pool was measured from dynamic data centred on the ^{99m}Tc signal and expressed as a percentage of the total blood granulocyte pool. Granulocyte migration was quantified on 24 h images using the ^{111}In signal. Granulocyte activation was measured as the percentage of cells showing a change in shape. The clearance rate of an inhaled aerosol of ^{99m}Tc-diethylenetriaminepenta-acetic acid (DTPA) was used as a marker of lung injury.

3. Pulmonary granulocyte pool, migration, activation and aerosol clearance, although highly variable in the patient groups, were, in general, elevated compared with the controls.

4. Granulocyte activation correlated with pulmonary granulocyte pool ($Rs=0.72$, $n=22$, $P<0.01$), while the $t_{1/2}$ of DTPA clearance correlated with migration ($Rs=-0.84$, $n=17$, $P<0.01$). Fifteen patients had an expanded pulmonary granulocyte pool, of whom six with no evidence of migration, had a normal DTPA clearance, while nine, who had an abnormal migration signal, had an accelerated DTPA clearance. The pulmonary granulocyte pool in these nine was significantly higher than in the six without a migration signal.

5. Activation of granulocytes results in delayed transit through the lung vasculature. With increasing margination, granulocytes migrate into the lung interstitium and injure the lung. An increased intravascular pool does not by itself lead to lung injury.

INTRODUCTION

The pulmonary kinetics of granulocytes have been widely investigated with respect to the role of these cells in acute lung damage [1–5]. Unlike capillaries elsewhere, lung capillaries have a calibre similar to or less than that of a granulocyte, which therefore undergoes mechanical deformation during transit through the pulmonary vascular bed [6–9]. Granulocytes have been seen, under direct observation, to undergo discrete periods of immobilization as they negotiate pulmonary capillaries [10]. Under pathological conditions, granulocytes may become activated as a result of exposure to inflammatory mediators, either in the circulation or locally at a site of inflammation [11]. This increases their mechanical stiffness, probably via effects on the cytoskeleton [8], and delays their lung transit. It has been widely suggested that such prolonged transit results in lung damage [1–5].

In the steady state, the number of granulocytes in the lung vasculature reflects the mean intrapulmonary vascular transit time. This population can be described as the pulmonary granulocyte pool (PGP) and, without implying a specific mechanism of intrapulmonary granulocyte transit, regarded as the sum of regional marginating and circulating granulocyte pools. It can be expressed as a fraction of the whole body total blood granulocyte pool (TBGP) by comparing the lung counts at 15 min after injection of ^{99m}Tc-labelled granulocytes with the first-pass counts on dynamic gamma-camera imaging, as recently described and validated [12]. Under normal circumstances it is reasonable to suppose that there is no significant migration of granulocytes into the extravascular space of the lung. As we have recently reported [13], extravascular migration into

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Abbreviations: DTPA, diethylenetriaminepenta-acetic acid; HMPAO, hexamethylpropylenamine oxime; IBD, inflammatory bowel disease; PGP, pulmonary granulocyte pool; ROI, region of interest; TBGP, total blood granulocyte pool.

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lung parenchyma can be quantified from gamma-camera images obtained 24 h after injection of ^{111}In-labelled granulocytes, when there are no longer any circulating labelled cells [14].

The PGP and extravascular migration can be measured separately using the same population of granulocytes, double labelled with ^{99m}Tc (for measuring PGP) and ^{111}In (for measuring migration) [15]. Thus, the superior counting statistics of ^{99m}Tc can be exploited for a dynamic study, while the greater cellular stability [14] and longer physical half-life of ^{111}In lend themselves to delayed imaging. With these techniques, therefore, we aimed to identify the separate roles of extravascular granulocyte migration and intravascular granulocyte pooling in the pathogenesis of lung injury by correlating them with granulocyte activation *in vivo*, measured by a simple but robust shape-change assay, and with the rate of clearance of inhaled ^{99m}Tc-diethylenetriaminepenta-acetic acid (DTPA) as an index of lung injury, in patients with extrapulmonary inflammatory diseases. These were inflammatory bowel disease (IBD) and systemic vasculitis, in which abnormalities of pulmonary granulocyte traffic have previously been demonstrated [16, 17], and graft versus host disease complicating bone marrow transplantation in which we have also frequently seen prominent lung activity early after injection of labelled granulocytes.

METHODS

Patients

A total of 23 non-smoking adult patients were studied, of whom 18 had systemic inflammation. They all received double-labelled granulocytes with quantification of PGP (based on ^{99m}Tc) and pulmonary extravascular granulocyte migration (based on ^{111}In). Six were referred for labelled granulocyte scintigraphy for the evaluation of suspected IBD or for follow-up of known IBD (three with ulcerative colitis, three with Crohn's disease; age range 22–50 years). All had abnormal focal accumulation of cells in the gastrointestinal tract indicating active IBD. Eight patients had clinically active systemic vasculitis (two with Behçet's disease, four with Wegener's granulomatosis, two with anti-neutrophilic cytoplasmic antibody-positive systemic vasculitis; age range 26–73 years), and four had abdominal graft versus host disease complicating bone marrow transplantation performed for chronic myeloid leukaemia (age range 33–52 years). Clinical management of the 18 patients was variable, although the vasculitic patients received prednisolone and azathioprine and all the bone marrow transplant recipients were on cyclosporin; they had also received total body irradiation and cyclophosphamide for conditioning before transplantation. Minor and variable abnormalities of lung function were documented in the vasculitic and transplant patients but in none of those with IBD.

In addition, five patients referred for suspected osteomyelitis from the Department of Surgery with a painful hip or knee prosthesis were included in the study. None gave a history of pyrexia during the month preceding referral, and, at the time of the study, each remained apyrexial and had a C-reactive protein level, leucocyte count and erythrocyte sedimentation rate in the normal range. They also all had negative granulocyte scans and a painful joint which was considered to be non-infected on clinical follow-up of at least 4 months. We therefore considered these five patients as negative for systemic inflammatory disease and used them as controls (patient controls). As none of these patient controls had a ^{99m}Tc-DTPA clearance study, we studied five healthy non-smoking volunteers (normal controls) to obtain normal clearance $t_{1/2}$ values.

Double ^{111}In and ^{99m}Tc granulocyte labelling

This technique and its potential advantages have recently been described and discussed in detail [15]. Briefly, 80–100 ml of venous blood was withdrawn through a 19-G butterfly cannula, anticoagulated with 20 ml of acid citrate dextrose (NIH Formula A), mixed with 10 ml of 6% hydroxyethyl starch solution and allowed to stand for 40–50 min to allow erythrocyte sedimentation. The leucocyte-rich plasma was removed and the granulocyte fraction isolated by discontinuous plasma–Percoll gradient centrifugation (50, 55 and 60%) using platelet-poor plasma. After isolation, the granulocytes were resuspended in 0.5 ml of platelet-poor plasma and labelled first with ^{111}In-tropolonate [18] and then with ^{99m}Tc-hexamethylpropylenamine oxime (HMPAO) [14] as follows. Nine to fifteen MBq of ^{111}In in 100 μl of tropolone were added to the granulocyte pellet which was incubated at room temperature for 5 min. Two millilitres of freshly prepared ^{99m}Tc-HMPAO (Ceretec, Amersham International plc, U.K.) (370–490 MBq) were added to the suspension and incubated for a further 10 min. The labelling was terminated by adding 20 ml of platelet-rich plasma. The labelled cells were then sedimented by centrifugation at 150 g for 5 min. The cell pellet was resuspended with 1.5 ml of cell-free plasma and reinjected into the patient. An aliquot was retained for the assessment of granulocyte activation (see below). Using this technique, each cell was labelled with both ^{111}In and ^{99m}Tc HMPAO; 148 (SD 12) MBq of ^{99m}Tc and 6.8 (0.2) MBq of ^{111}In were injected.

Granulocyte kinetic studies

All studies were performed with a Starcam (International General Electric) large field-of-view gamma-camera on-line to a dedicated computer (Starimager; International General Electric) for sub-

sequent analysis. The labelled cells were injected via a 19-G butterfly cannula as a bolus and a 30-min dynamic study was acquired in the ^{99m}Tc gamma photopeak. Regions of interest (ROI) were placed over the right ventricle and right lung and time–activity curves generated for quantification of PGP, as previously described [12]. Conventional planar scans were recorded on the ^{111}In photopeak at 24 h after injection. One of the posterior views encompassed the middle and lower lobes of the lungs and the pelvis. ROIs were drawn over the right lung and posterior iliac bone marrow (adjacent to the sacroiliac joint) and the average count rate per pixel was calculated, corrected for ^{111}In physical decay and expressed in relation to the acquisition time, size of ROI and injected activity as c.p.m. MBq^{-1} pixel^{-1}. We have previously shown that in normal subjects the chest and iliac bone marrow ^{111}In uptake on 24 h ^{111}In-granulocyte scans correlated with each other [13]. Assuming no granulocytes migrated into the lung interstitium in these subjects, the interstitial lung signal in the current study was calculated from (right lung ROI uptake) $-0.23 \times$ (iliac bone marrow ROI uptake) [13] and used as an index of pulmonary granulocyte migration on the grounds that by 24 h there are no longer any intravascular labelled cells [14, 19].

Measurement of granulocyte activation

The activation of granulocytes was quantified using a shape-change technique which measures granulocyte polarization consequent upon cytoskeletal assembly [20]. Measured activation represents the effects of activating stimuli present *in vivo* before granulocyte isolation and *ex vivo* as a result of the preparative method. A 0.1 ml aliquot of granulocyte suspension in plasma was withdrawn from the cell pellet isolated for labelling and transferred to a 15 ml conical polyethylene tube. It was then made up to 10 ml with Hanks' buffered saline solution free of Ca^{2+} and Mg^{2+}. While making up the volume, cells were resuspended gently with a plastic Pasteur pipette. They were then centrifuged at 150*g* for 4 min and the supernatant decanted. The cell pellet was resuspended in 0.5 ml of Hanks' buffered saline solution with Ca^{2+} and Mg^{2+} and incubated for 10 min at 37°C. The cells were then fixed with 0.5 ml of 2% glutaraldehyde. Activation was quantified as the percentage of cells showing a change in shape on microscope counting. The test was not performed in one of the patient controls. The effect of labelling itself was investigated in samples from 10 subjects (four healthy controls and six of the patients with systemic inflammation) by comparing shape change in aliquots withdrawn from the cell pellet before and immediately after double labelling.

^{99m}Tc-DTPA inhalation clearance studies

Alveolocapillary permeability was measured after the completion of 24 h ^{111}In imaging in 17 of the 18 patients with systemic inflammation. With the gamma-camera positioned posteriorly over the chest and upper abdomen, the supine patient inhaled ^{99m}Tc-DTPA aerosol via an InterSurgical jet inhaler for 4 min. Lung clearance was monitored for 60 min. Lung background activity was subtracted using an ROI over the liver and a separate intravenous injection of ^{99m}Tc-DTPA, as described previously [21]. Each background-corrected clearance curve was displayed on a logarithmic ordinate and fitted by least squares to a mono-exponential or bi-exponential function, depending on which was closer on maximum likelihood criteria [22]. The overall $t_{1/2}$ was calculated as the time to half-maximum lung count rate, irrespective of the number of exponentials present.

Statistics

Non-parametric statistics were used throughout: Wilcoxon rank sum and signed rank sum methods for comparison of independent and paired samples, respectively, and Spearman rank correlation coefficient (*Rs*) for associations between variables. A *P* value of less than 0.05 was taken to represent statistical significance.

The study was approved by the local Ethics Committee of Hammersmith Hospital and the Administration of Radioactive Substances Advisory Committee of the Department of Health. Informed consent was obtained from patients and volunteers.

RESULTS

There was no significant change in granulocyte activation after labelling in comparison with activation present before labelling; i.e. immediately after isolation from blood. Thus, average cell activation in four normal volunteers was 9.8% before labelling and 10.1% after labelling, and in six patients with systemic inflammatory conditions it was 32% before and 34% after labelling.

Values for PGP, granulocyte activation, migration index and $t_{1/2}$ of ^{99m}Tc-DTPA clearance are illustrated in Fig. 1. The migration index tended to be bimodal in each of the three patient groups. The above variables were significantly different compared with patient controls in patients with vasculitis (except for migration) and in bone marrow transplant recipients (except for migration and DTPA $t_{1/2}$). In IBD, on the other hand, none of the variables were significantly different compared with patient controls.

Combining patients from all groups, there were strong associations between the $t_{1/2}$ of ^{99m}Tc-DTPA clearance and the migration index ($Rs = -0.84$, $n = 17$, $P < 0.01$) and between granulocyte activation and PGP ($Rs = 0.72$, $n = 22$, $P < 0.01$) (Fig. 2). Significant but weaker associations were noted between migration and PGP ($Rs = 0.58$, $n = 23$), and between migration and activation ($Rs = 0.52$, $n = 22$). The associations between ^{99m}Tc-DTPA clearance $t_{1/2}$

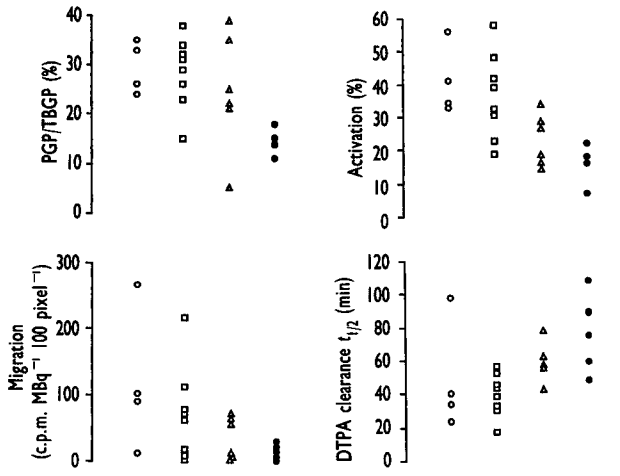

Fig. 1. Values for PGP (as a percentage of the TBGP), granulocyte activation, extravascular migration and pulmonary ^{99m}TC-DTPA aerosol clearance in patients after bone marrow transplantation (○), with systemic vasculitis (□), with active IBD (△) and in patient controls (●).

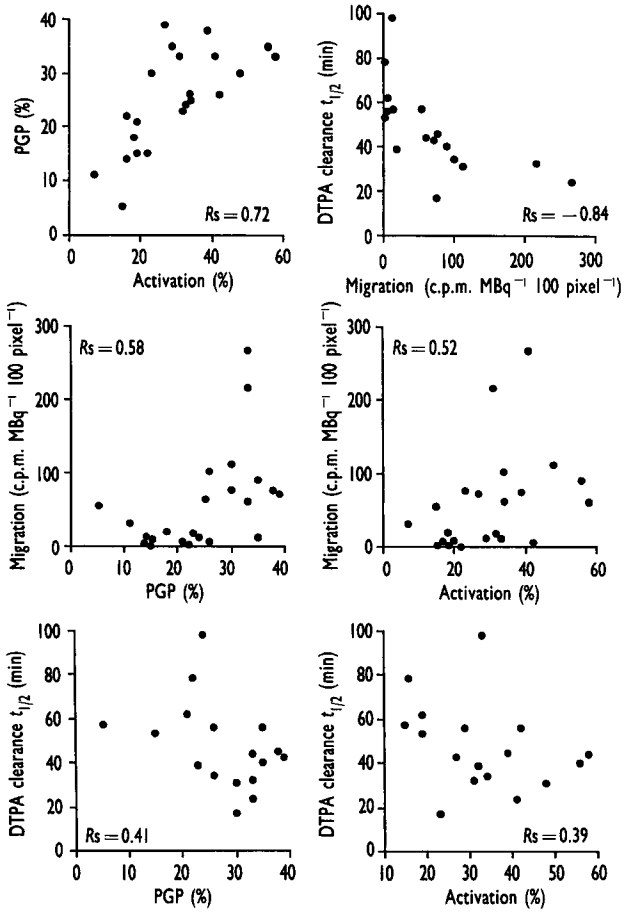

Fig. 2. Relationships between pulmonary granulocyte pooling, granulocyte activation, extravascular migration and pulmonary Tc-99m-DTPA aerosol clearance in all patients. Spearman rank correlation coefficients are shown.

and PGP ($Rs = -0.41$, $n = 17$), and between ^{99m}Tc-DTPA clearance $t_{1/2}$ and granulocyte activation ($Rs = -0.39$, $n = 17$), were weak and failed to reach significance.

The ^{99m}Tc-DTPA clearance was mono-exponential in all the normal volunteers and patients with IBD but was bi-exponential in three bone marrow transplant recipients and in four patients with vasculitis. The average overall clearance $t_{1/2}$ was 35 (range 31–44) min in the seven patients with bi-exponential clearances, significantly shorter compared with that in the patients with mono-exponential clearances, in whom it was 57 (range 17–98) min ($P < 0.01$). Furthermore, the average migration signal in the seven patients with bi-exponential clearances was 1.23 (range 0.18–2.66) c.p.m. MBq^{-1} pixel^{-1}) significantly higher than in the 10 with mono-exponential clearances, in whom it averaged 0.32 (range 0.02–0.76) c.p.m. MBq^{-1} pixel^{-1} ($P < 0.01$).

In view of the tendency towards a bimodal distribution of migration indices in the patients with systemic inflammation, we next examined the differences between patients without and those with significant migration. The 17 patients with a complete data set comprised: (i) six with an expanded PGP (>20%) but a migration index within the normal range (<0.2 c.p.m. MBq^{-1} pixel^{-1}); (ii) nine with an expanded PGP and an increased migration index; (iii) one with increased migration but normal PGP; and (iv) one with normal pulmonary granulocyte kinetics. The nine patients with increased migration and increased PGP had significantly higher values of PGP and ^{99m}Tc-DTPA clearance rates than the six with a raised PGP but no migration signal (Table 1). Their granulocyte activation, however, was not significantly higher. Patients with an expanded PGP but no increased migration had ^{99m}Tc-DTPA clearance rates that were not significantly different from normal controls.

DISCUSSION

In the patient control group, the PGP was quite close to the lung blood volume expressed as a fraction of total blood volume [23], suggesting that, although a small number of cells may undergo prolonged retention, there is normally only minimal granulocyte retention in the lung vasculature. This is consistent with quantitative studies of the whole body distribution of granulocytes in normal subjects [24]. In contrast, as we have shown previously [12], PGP may be increased in patients with systemic inflammation. Less attention has been paid to extravascular granulocyte migration in the lung, possibly because it is difficult to quantify, even by bronchopulmonary lavage. We have developed a technique for quantifying migration [13] based on the residual ^{111}In signal in the lung after 24 h when it is reasonable to assume that labelled cells in the lung will all have migrated. Although some authors,

Table 1. Comparison of PGP, granulocyte activation, granulocyte migration and ^{99m}Tc-DTPA aerosol clearance rate in patients with an expanded PGP between those in whom migration was ($n=9$) or was not ($n=6$) detected. Average values with ranges are shown. Abbreviations: NA, not applicable; NS, not significant.

	Non-migrators	P (versus controls)	Migrators	P (versus non-migrators)
Migration (c.p.m. MBq^{-1} pixel^{-1})	0.094 0.02–0.18	NA	1.19 0.61–2.66	NA
PGP (%)	25 21–35	NA	33 26–39	<0.05
Activation (%)	29 16–42	NS	40 23–58	NS
$t_{1/2}$ DTPA (min)	65 39–98	NS	34 17–45	<0.01

directly observing pulmonary capillaries through skin windows, have described the presence of granulocytes sequestered in pulmonary capillaries for periods of many minutes to hours [7–10], these must represent an increasingly small minority of the pulmonary granulocyte population, even in patients with an expanded PGP, otherwise in due course a very large fraction of the whole body TBGP would accumulate in the pulmonary vasculature.

Granulocyte activation was assessed by the simple, rapid, robust and well-validated method of determining the proportion of cells exhibiting shape-change during 10 min incubation at 37°C [20]. This assay assesses the effect of activating stimuli upon polarization of cytoskeletal elements and is therefore of direct relevance for assessing whether re-infused granulocytes are likely to be retarded during passage through the pulmonary capillaries because of reduced deformability [25, 26]. Furthermore the assay has the advantage that activation is assessed on an all-or-nothing basis for individual cells. Other assays of granulocyte activation, such as *N*-formylmethionyl-leucyl-phenylalanine stimulated superoxide production or flow cytometric demonstration of increased CD11b expression, reflect a continuous property of the whole population of cells and their relevance to the ability of granulocytes to deform and traverse the pulmonary circulation is uncertain. Granulocytes were activated in several patients with systemic inflammation. The mechanisms of activation have not been addressed in this study. In several studies based on shape-change techniques or flow cytometry, increased activation of granulocytes has been shown in vasculitis [27], IBD [28] renal transplant recipients [29] and vascular graft recipients [30], probably resulting from circulating and locally generated cytokines in inflamed tissue [11, 28, 31]. Activation in our controls was higher than recorded by Haslett et al. [20]; we ascribe this to cell manipulation *in vitro*. The effect of Percoll in this respect has recently been shown to be less than that of other isolation techniques [32].

PGP correlated with granulocyte activation, implying that activated granulocytes have a longer transit time through lung vessels as compared with non-activated cells. Biophysical data obtained from experiments *in vitro* [9, 25] and experimental data *in vivo* [26] support this suggestion. The activated stiffened cells transit the pulmonary vessels more slowly than normal cells and make the PGP dependent upon their proportion in the total granulocyte population. Activation of cells during labelling also results in a delay in granulocyte transit through the lung vasculature [33] and this may be related to mechanical stiffness. Nevertheless, the kinetics of the granulocytes are different in this form of 'artefactual' hold-up, with relatively rapid clearance of pulmonary activity, a very low recovery in circulating blood and prominent uptake of cells by the liver [33]. Furthermore, there was no significant difference between pre- and post-labelling activation over a wide range of values. In addition, we did not observe any cases of artefactual lung sequestration. We propose that granulocyte activation is primarily related to circulating factors or exposure of the cells to cytokines in the inflammatory lesion.

Lung parenchymal damage has been widely quantified from the clearance of inhaled ^{99m}Tc-DTPA, which is significantly increased after inhalation of fire smoke [34] and in patients with interstitial lung disease [35], adult respiratory distress syndrome [36] and sarcoidosis [37]. A bi-exponential clearance is generally thought to reflect severe damage [38]. Alveolocapillary permeability has not been widely investigated in systemic inflammation, although Lecouffe et al. [39] found an accelerated ^{99m}Tc-DTPA clearance in Crohn's disease. In the current study we observed a significant correlation between the migration signal and the $t_{1/2}$ of ^{99m}Tc-DTPA clearance. Furthermore, patients with bi-exponential ^{99m}Tc-DTPA clearances had significantly higher migration signals than patients with mono-exponential clearances. However, several patients who had an increased PGP and activated granulocytes had little evidence of parenchymal migration. As a group, these patients had no evidence of lung injury insofar as their ^{99m}Tc-DTPA clearance was not significantly different from normal subjects. Unlike the systemic vascular bed, in which granulocytes gain access to the extravascular space by migrating across post-capillary venules, granulocytes in the lung migrate through the capillary endothelium [40]. Thus, consistent with clinical

[41] and animal data [40, 42, 43], it seems likely that granulocytes have to migrate across this endothelial barrier to cause damage to lung parenchyma. Although a migration signal was absent in an appreciable number of patients with increased PGP, the latter was significantly higher in the presence of migration, consistent with the hypothesis that migration occurs above a critical level of pulmonary granulocyte margination. Our data does not, however, exclude the possibility that intravascular granulocytes, adherent to the pulmonary endothelium, may cause endothelial damage [44], since the rate-limiting barrier for the lung clearance of inhaled ^{99m}Tc-DTPA is the alveolar epithelium rather than the vascular endothelium. Thus, indices of lung injury based, for example, on the accumulation of radiolabelled protein in the pulmonary interstitial space, could potentially be elevated in the presence of intravascular granulocyte accumulation without migration.

In conclusion, systemic inflammation is associated with increased granulocyte activation and pulmonary granulocyte pooling. Our data are consistent with the hypothesis that a further increase in pooling is associated with migration. Interstitial lung damage does not, however, result from intravascular pooling by itself but also requires extravascular migration. A sequence can therefore be proposed in which lung injury is the end result of granulocyte activation, leading to pulmonary granulocyte margination, increasing margination and extravascular migration. Further work is required to more clearly define the aetiological role of pulmonary granulocytes in diffuse lung damage.

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