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Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein

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ABSTRACT: ***Streptococcus pyogenes*** **of the M1 serotype can cause streptococcal toxic shock syndrome commonly associated with acute lung injury. The aim of the present study was to investigate the role of neutrophils and their secretion products in M1 protein-induced lung damage.**

The degranulation of neutrophils by M1 protein was studied in whole blood using marker analysis for individual granule subsets. In mice, M1 protein was injected intravenously and the lung damage was assessed by histology, electron microscopy, cell count in bronchoalveolar lavage fluid and analysis of lung vascular permeability. Comparisons were made in mice with intact white blood count, neutropenic mice and neutropenic mice injected with the secretion of activated neutrophils.

In whole blood, M1 protein forms complexes with fibrinogen that bind to β_2-integrins on the neutrophil surface, resulting in degranulation of all four subsets of neutrophil granules. Intravenous injection of M1 protein into mice induced neutrophil accumulation in the lung, increase in vascular permeability and acute lung damage. Depletion of neutrophils from the circulation completely abrogated lung injury and vascular leakage. Interestingly, the lung damage was restored by injecting neutrophil secretion.

The present data suggest that neutrophil granule proteins are directly responsible for lung damage induced by the streptococcal M1 protein.

KEYWORDS: **Lung injury, M1 protein, neutrophil granule proteins, *Streptococcus pyogenes***

Polymorphonuclear leukocytes (PMNs) are the earliest immune cells to be recruited to the site of injury or infection, and release a wide array of granule proteins that contribute to host defence and tissue repair [1]. However, in some situations there is a misdirected activation of the immune system, which may in itself give rise to host tissue damage. For example, bacterial infections and septicaemia involve immune cell activation that could potentially lead to lung injury. The contribution of PMNs to vascular dysfunction in response to bacterial infections is controversial, and may depend on the specific pathogen involved. However, in experimental mouse models of septicaemia, neutropenic mice often show reduced lung damage compared with normal mice, and studies involving inhibitors of neutrophil components or PMN granule protein knockout mice point towards the involvement of PMNs and their secretion products in the initiation and progress of the lung injury [2, 3].

Streptococcus pyogenes is a significant human pathogen causing a wide panoply of diseases, from uncomplicated infections to life-threatening conditions such as streptococcal toxic shock syndrome (STSS), which is characterised by hypotension and multiple organ failure. The M protein is a major surface protein and, due to its antiphagocytic function, is a virulence factor of *S. pyogenes*. Of the >80 serotypes, the M1 serotype is predominantly associated with fatal STSS [4]. It has been recently reported that M1 protein released from the bacterial surface, spontaneously or by the action of proteases, forms complexes with fibrinogen, which in turn activate PMNs to liberate heparin-binding protein (HBP) [5]. Notably, previous studies have shown that HBP is a crucial mediator of PMN-induced permeability increase in inflammation [6] and, hence, it is tempting to speculate that HBP is critically involved in *S. pyogenes*-induced lung injury. Recent studies revealed further virulence mechanisms of M1 protein, all of which may contribute to the lung damage observed in STSS. These mechanisms include cytokine [7] and tissue factor [8] release from monocytes, as well as chemokine expression in epithelial cells [9].

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Moreover, M1 protein was shown to induce activation of T-cells [10] and platelets [11], which results in thrombus formation.

The present study was undertaken in order to investigate the importance of PMN activation in response to M1 protein–fibrinogen complexes in the pathogenesis of M1 protein-induced lung damage. The data indicate direct proof for the almost exclusive role of PMN degranulation in the onset of M1 protein-mediated lung injury, which may serve as a primary therapeutic target.

MATERIALS AND METHODS

PMN activation by M1 protein

M1 protein (1 μg·mL^{-1}), generated as described previously [5], was added to human whole blood in the presence or absence of the peptides Gly-Pro-Arg-Pro or Gly-His-Arg-Pro (1 mM; Bachem, Bubendorf, Switzerland) or the CD18 antibody IB4 (10 μg·mL^{-1}). Some samples were treated with protein H (1 μg·mL^{-1}) from *S. pyogenes* instead of M1 protein. After incubation at 37°C for 30 min the samples were centrifuged ($300 \times g$, 15 min) and the supernatant was analysed for myeloperoxidase (MPO) and matrix metalloproteinase (MMP)-9. PMNs in the pellet were stained with antibodies to CD16 (a marker for secretory vesicles; Becton Dickinson, Franklin Lakes, NJ, USA), CD11b (a marker for secretory vesicles and tertiary granules; Pharmingen, Franklin Lakes, NJ, USA), CD66b (a marker for secondary granules; Immunotools, Friesoythe, Germany), and CD63 (a marker for primary granules; Eurobiosciences, Friesoythe, Germany) and analysed by fluorescence-activated cell sorting (FACS).

Animal experiments

Balb/c mice of either sex, weighing ~20 g, were used for *in vivo* experiments. Mice were anaesthetised by isoflurane inhalation followed by ketamine/xylazine *i.p.*, and a catheter was placed in the left jugular vein. Mice were intravenously injected with 15 μg M1 protein in PBS, and were sacrificed 30 min or 4 h later by an overdose of anaesthetic. PMN depletion was induced through *i.p.* treatment with monoclonal antibody (mAb) RB6-8C5 (250 μg per mouse) 12 h before injection of M1 protein [12]. Neutropenia was confirmed on the day of experiment by manual blood count. Monocytes were eliminated 18 h prior to experimentation by *i.v.* injection of 0.2 mL of clodronate liposomes into the lateral tail vein, as described previously [13]. Clodronate was a gift from Roche (Mannheim, Germany) and was incorporated into liposomes, as previously described [14]. Depletion of monocytes was monitored by FACS analysis using antibodies to Gr1 and F4/80. To substitute for PMN granule products in neutropenic animals, 300 μL of human PMN secretion were injected in parallel with M1 protein. This value was based on the assumption that the average mouse blood contains about 3×10^6 PMNs that would be activated in the presence of M1 protein.

In separate experiments, M1 protein–fibrinogen precipitate was formed *ex vivo* and injected intravenously. M1 protein (20 μg) was added to fibrinogen (6 mg) in distilled water and incubated for 10 min. After centrifugation, the pellet was re-suspended in 100 μL PBS and injected into the jugular vein. Mice stimulated in this way were subjected to treatments similar to those directly injected with M1 protein. All animal experiments were approved by the local ethical committee (Northern Stockholm Animal Ethics Committee, Sweden) for animal experimentation.

PMN secretion

Human PMNs were isolated from fresh blood of healthy donors using Polymorphprep (Nycomed Pharma, Oslo, Norway) according to the manufacturer's instructions. PMNs were resuspended in Dulbecco's modified Eagle medium (DMEM) at 10×10^6 cells·mL^{-1} and PMN secretion was obtained by antibody cross-linking of CD18 as described previously [15].

In separate experiments, murine whole blood was obtained by cardiac puncture and PMNs were isolated using NycoPrep Animal (Nycomed Pharma). PMNs were resuspended in DMEM and incubated for 30 min at 37°C. Cells were spun down and the supernatant was used as control secretion. Thereafter, PMNs were again resuspended in DMEM and incubated with M1 protein–fibrinogen precipitate. After 30 min, PMNs were spun down and the supernatant was used as M1 secretion.

Bronchoalveolar lavage and vascular permeability assay

After exsanguination *via* the vena cava inferior, the trachea was catheterised and the left lung was lavaged three times with 500 μL PBS. Leukocytes in the bronchoalveolar lavage (BAL) fluid were manually counted and the protein concentration was assessed using a standard protein assay (BioRad, Hercules, CA, USA). BAL protein concentration and the wet/dry weight ratio were used as indicators of plasma exudation. To obtain the wet/dry weight ratio, excised lungs were weighed, dried overnight at 60°C and weighed again. In mice subjected to injection of pre-formed M1 protein–fibrinogen precipitate, Evans blue (EB) dye was used to assess vascular leakage [16]. EB (50 mg·kg^{-1}) was administered *i.v.* and dye extravasation used to assess change in vascular permeability. At the end of the experiment, the pulmonary circulation was flushed with PBS and EB was extracted from homogenised lung tissue by incubating in formamide for 24 h at 60°C. The optical density of the supernatant and of serum was measured at 620 nm and EB–albumin extravasation was expressed as microlitre serum equivalents per gramme of lung tissue.

Histology and electron microscopy

After completion of the experiment, one part of the right lung was fixed in formalin, embedded in paraffin and stained with Mayer's haematoxylin and eosin for histological examination [5]. Another part of the lung was prepared for scanning electron microscopy as described previously [5].

Analysis of PMN degranulation

The release of the primary granule specific enzyme MPO from PMNs after incubation of whole blood with M1 protein was quantified as previously described by SUZUKI *et al.* [17].

Qualitative release of the secondary and tertiary granule enzyme MMP-9 from PMNs was analysed by Western blot as described previously [18]. MMP-9 activity was quantified using the SensoLyte MMP-9 assay kit (Anaspec, San Jose, CA, USA). The fluorescent product was measured at 520 nm using

a fluorescence plate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

Statistics

Data were analysed *via* ANOVA, followed by Tukey's HSD test if the overall *F*-ratio was significant. The results are presented as individual values or mean±SD. A p-value <0.05 was considered significant.

RESULTS

M1 protein–fibrinogen complexes induced degranulation of PMNs

Previous work has shown that M1 protein, when added to human blood, forms complexes with fibrinogen [5]. In turn, these complexes are capable of activating PMNs and thereby induce the release of granule proteins from internal stores [5]. To assess whether all four PMN granule subsets are liberated in response to M1 protein, the current authors incubated human whole blood with M1 protein. Mobilisation of PMN granules was recorded by FACS analysis, gating on the PMN in the forward/side scatter allowing the specific analysis of upregulation of marker proteins of granule subsets. In the presence of M1 protein an upregulation of CD16, CD11b, CD66b and CD63 was found, indicative of the mobilisation of secretory vesicles and tertiary, secondary and primary granules. Similarly, a strong increase in the primary granule marker protein MPO and in the secondary and tertiary granule marker MMP-9 could be detected in the plasma (figs 1 and 2). Fibrinogen binds to PMNs *via* β_2-integrins and it has been shown that the Gly-Pro-Arg-Pro peptide effectively blocks adhesion of activated PMNs to fibrinogen [19]. Therefore, experiments were performed in the presence of the Gly-Pro-Arg-Pro peptide or the control peptide Gly-His-Arg-Pro. It was found that treatment with Gly-Pro-Arg-Pro blocks the release of all marker proteins from M1 protein-stimulated PMN (figs 1 and 2), while the control peptide had no effect. The β_2-integrin antibody IB4 had similar effects to those of the Gly-Pro-Arg-Pro, further supporting the crucial role of β_2-integrins in PMN degranulation. *S. pyogenes* not only sheds M1 protein but also other surface proteins. The current authors investigated the specificity of the proposed mechanism and incubated whole blood with protein H, another surface protein isolated from the M1 serotype of *S. pyogenes*. However, protein H did not result in release of MPO or MMP-9 from PMNs (fig. 2).

Intravenous injection of M1 protein into mice caused neutrophil-dependent lung damage

In order to characterise the role of PMN activation in the responses to M1 protein–fibrinogen complexes *in vivo*, mice were injected with M1 protein *i.v.* (15 μg per mouse) and followed for 30 min. After exsanguination, the lungs were removed and analysed by light microscopy and scanning electron microscopy. Compared with mice treated with vehicle only, M1 protein injection induced severe lung damage, depicted by haemorrhage, deposition of fibrinogen aggregates and swelling of the alveolar membrane (fig. 3). Moreover, BAL was carried out in order to monitor the inflammatory response. To this end, when BAL was analysed with respect to leukocyte and protein content, the number of cells in the BAL fluid, most of which were PMNs, was clearly increased 30 min after injection of M1 protein. This suggests an involvement of these cells in the response to M1 protein (fig. 4a). Moreover, the current authors assessed the protein concentration in the BAL fluid and the wet/dry weight ratio of the lungs. Both parameters, when enhanced, indicate an increase in vascular permeability in the pulmonary circulation. Intravenous injection of M1 protein significantly increased these two values (fig. 4c and e). Therefore, the extravasation of PMNs to the alveolar space was associated with leakage of plasma from the lung vasculature, which conforms to the documented link between neutrophil recruitment to inflammatory loci and increase in vascular permeability [20].

In the next series of experiments, the contribution of PMNs to M1 protein-induced lung damage was analysed by removing PMNs from mice on *i.p.* treatment with mAb RB6-8C5. Antibody injection resulted in a total neutropenia (<500 cells·μL^{-1} and $<20\%$ of basal PMN count), which was sustained throughout the experimental procedure. In PMN-depleted mice, no destruction of the lung tissue was seen after M1 protein injection (fig. 3). Moreover, the lung vascular permeability was not significantly altered compared with the control mice (fig. 4c and e). Similar results were found when mice were treated with M1 protein for 4 h (fig. 4b, d and f), suggesting that PMNs may be involved not only in the immediate response to M1 protein but also in the sustained lung destruction. These relationships were further established in a second set of experiments where pre-formed M1 protein–fibrinogen complexes were injected. Injection of the M1 protein–fibrinogen precipitate resulted within 30 min in enhanced vascular permeability. With regard to the response to injection of M1 protein alone, the permeability increase was dependent on the presence of PMNs as enhanced protein extravasation was largely prevented in PMN-depleted mice (fig. 5). Interestingly, these data suggest that the M1 protein–fibrinogen complex, rather than M1 protein itself, is central to the pathogenesis of the vascular derangement following *S. pyogenes* infection. Collectively, these observations clearly imply an imperative role of PMNs in the lung injury caused by M1 protein.

It has previously been shown that M1 protein activates monocytes to express pro-inflammatory cytokines and tissue factor [7, 8]. To address the possible contribution of monocytes to M1 protein-induced lung damage, monocytes were depleted by intravenous application of clodronate liposomes. Instillation of M1 protein in these mice did not reduce the lung damage compared with mice with intact white blood count, indicating a minor contribution of monocytes (figs 3 and 4).

PMNs contributed to lung injury via release of PMN granule proteins

PMNs may contribute to lung tissue dysfunction and altered vascular permeability through different mechanisms, *e.g.* release and production of cytokines [21], generation of reactive oxygen species (ROS) [22] or exocytosis of pre-formed granule proteins [3, 6]. Since the present results demonstrate that the M1 protein–fibrinogen complex is a powerful inducer of PMN degranulation, it was of interest to investigate the impact of PMN secretion products on lung vascular function. PMN secretion (300 μL per mouse) obtained from human PMNs after antibody cross-linking of CD18 was injected intravenously into neutropenic mice, together with M1 protein or M1 protein–fibrinogen precipitate. Injection of the PMN

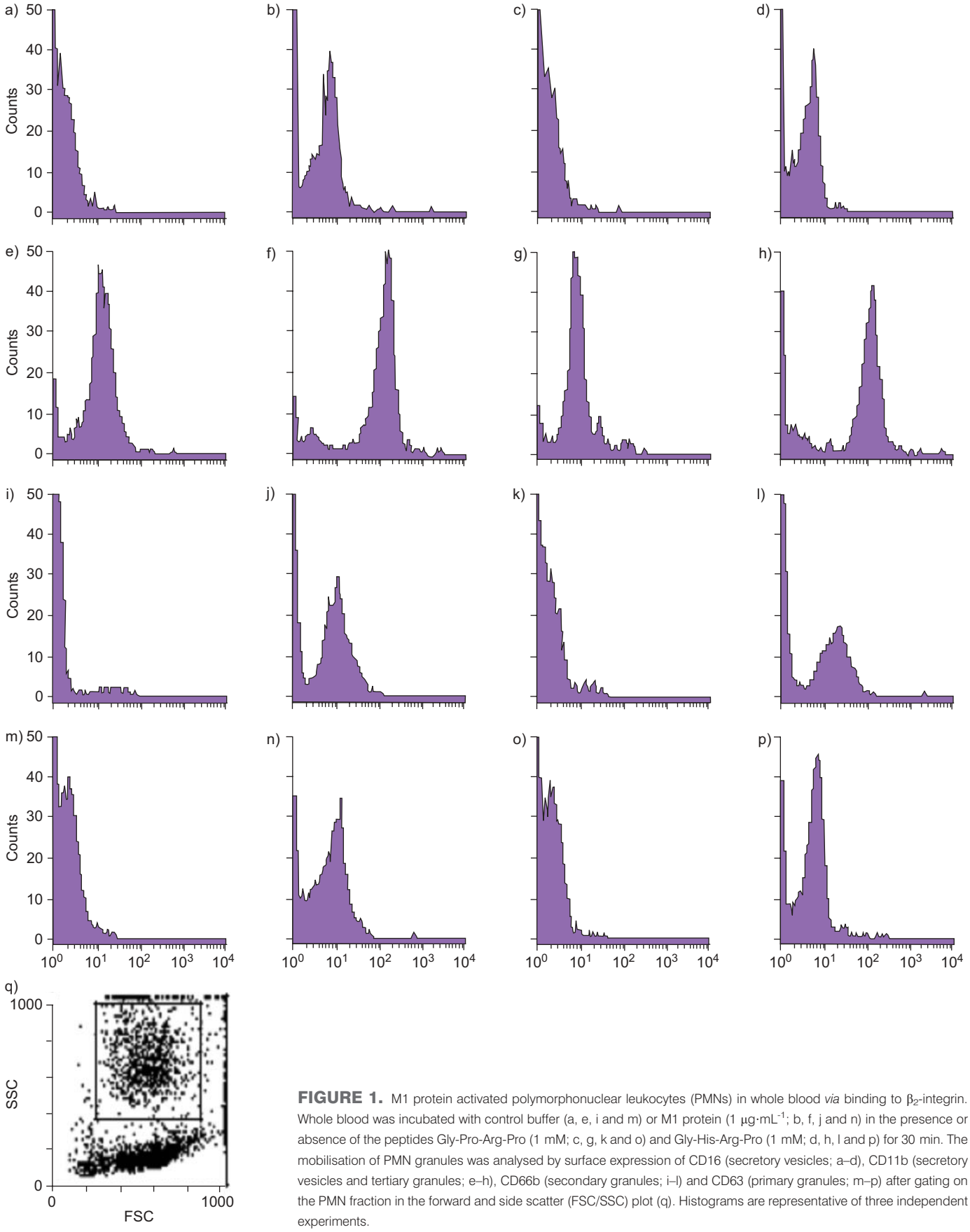

FIGURE 1. M1 protein activated polymorphonuclear leukocytes (PMNs) in whole blood *via* binding to β_2-integrin. Whole blood was incubated with control buffer (a, e, i and m) or M1 protein (1 $\mu g \cdot mL^{-1}$; b, f, j and n) in the presence or absence of the peptides Gly-Pro-Arg-Pro (1 mM; c, g, k and o) and Gly-His-Arg-Pro (1 mM; d, h, l and p) for 30 min. The mobilisation of PMN granules was analysed by surface expression of CD16 (secretory vesicles; a–d), CD11b (secretory vesicles and tertiary granules; e–h), CD66b (secondary granules; i–l) and CD63 (primary granules; m–p) after gating on the PMN fraction in the forward and side scatter (FSC/SSC) plot (q). Histograms are representative of three independent experiments.

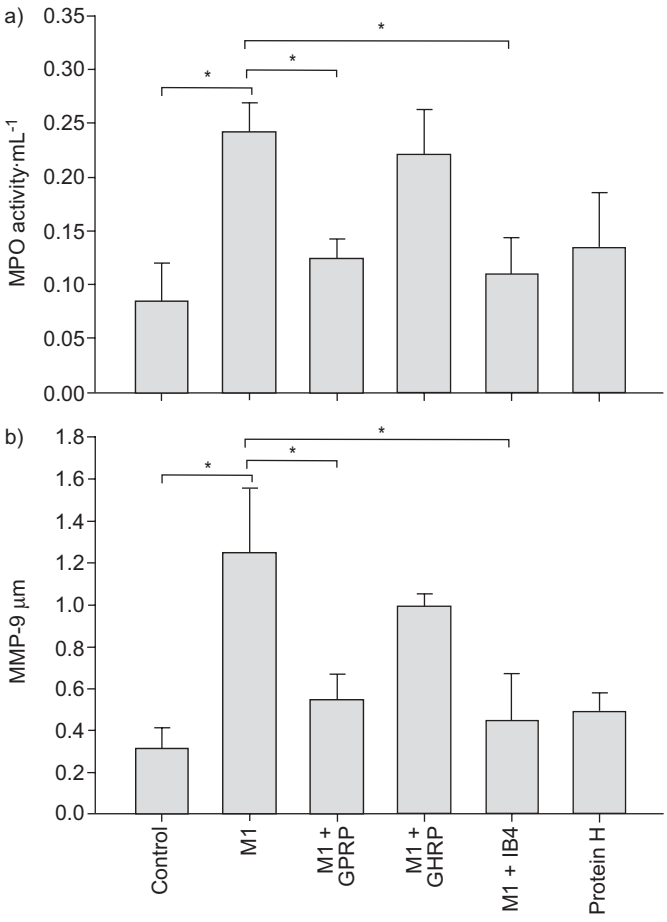

FIGURE 2. M1 protein activated polymorphonuclear leukocytes (PMNs) in whole blood *via* binding to β_2-integrin. Whole blood was incubated with M1 protein (1 μg·mL^{-1}) in the presence or absence of the peptides Gly-Pro-Arg-Pro (GPRP; 1 mM) or Gly-His-Arg-Pro (GHRP; 1 mM), or monoclonal antibody IB4 (10 μg·mL^{-1}) for 30 min. Alternatively, whole blood was incubated with protein H (1 μg·mL^{-1}). a) Release of the primary granule marker myeloperoxidase (MPO) was assessed by spectrophotometric analysis, and b) release of the secondary and tertiary granule marker matrix metalloproteinase (MMP)-9 was analysed by fluorometric analysis. Data are expressed as mean±SD of three independent experiments. *: $p<0.05$.

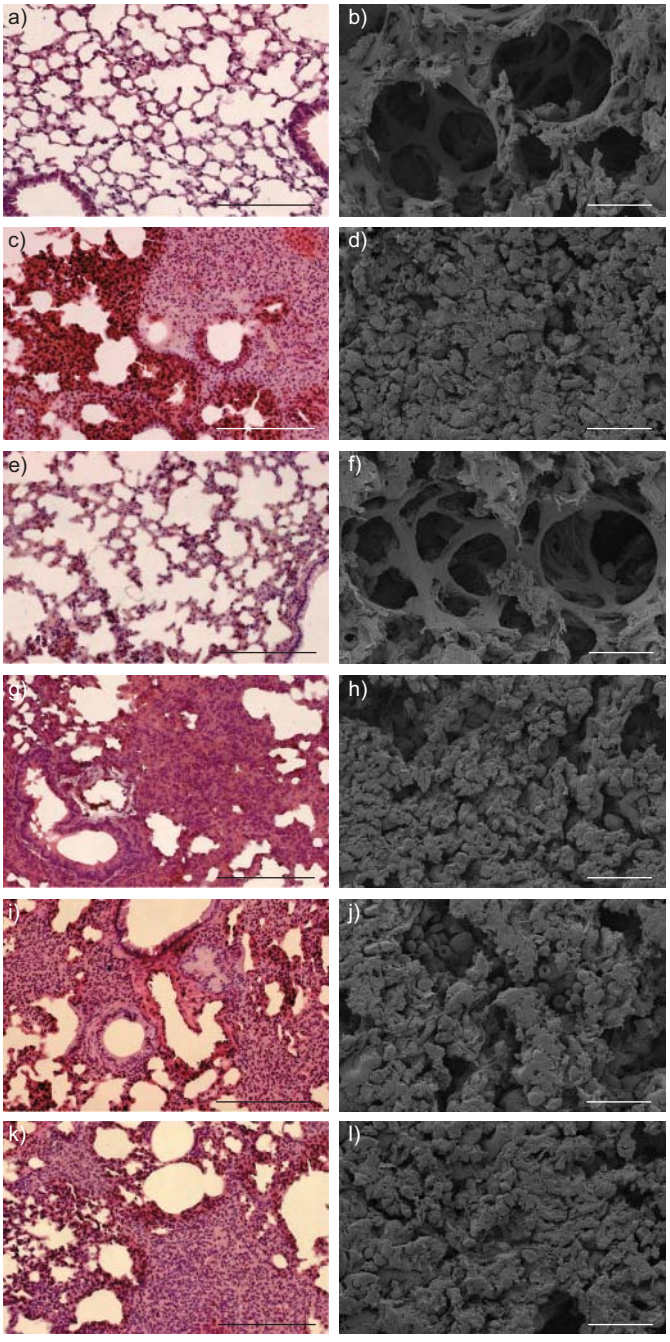

FIGURE 3. M1 protein-induced lung damage in mice was mediated by polymorphonuclear leukocyte (PMN) granule proteins: morphological data. Light microscopy (a, c, e, g, i and k) and scanning electron microscopy (b, d, f, h, j and l) of murine lung sections are exemplified. The figure shows representative micrographs of lungs from mice injected intravenously with buffer alone (a and b) or M1 protein (30 min, 15 μg; c and d). In addition, animals were rendered neutropenic (e and f) and injected with PMN secretion (g and h) prior to injection of M1 protein. Moreover, mice were depleted of monocytes before injection of M1 protein (i and j). Finally, mice received PMN secretion only (k and l). Scale bars=250 μm (a, c, e, g, i and k) and 25 μm (b, d, f, h, j and l).

secretion caused a similar deleterious lung injury and enhanced permeability in neutropenic mice, as seen after injection of M1 protein or M1 protein–fibrinogen precipitate in mice with intact white blood cells (figs 3–5). A similar response was found when PMN secretion was injected in the absence of M1 protein (figs 3 and 4). In further experiments, the current authors injected murine PMN secretion obtained from isolated blood PMNs activated with M1 protein–fibrinogen complexes. The murine PMN secretion induced vascular leakage to an extent similar to that of the human secretion (fig. 6). Since injection of the PMN secretion completely mimicked the lung damage induced by M1 protein, it can be suggested that PMN degranulation constitutes a final critical step within the chain of events triggered by M1 protein that eventually leads to lung injury. Western blot analysis revealed the presence of elastase, LL-37, MMP-9 and albumin, indicative of the release of primary, secondary and tertiary granules, as well as secretory vesicles, in the PMN secretion [23]. The current authors were unable to detect ROS in the PMN secretion, which is also to be expected in view of the short lifespan of these elements. Cytokines and chemokines are not stored in PMN granules [24]

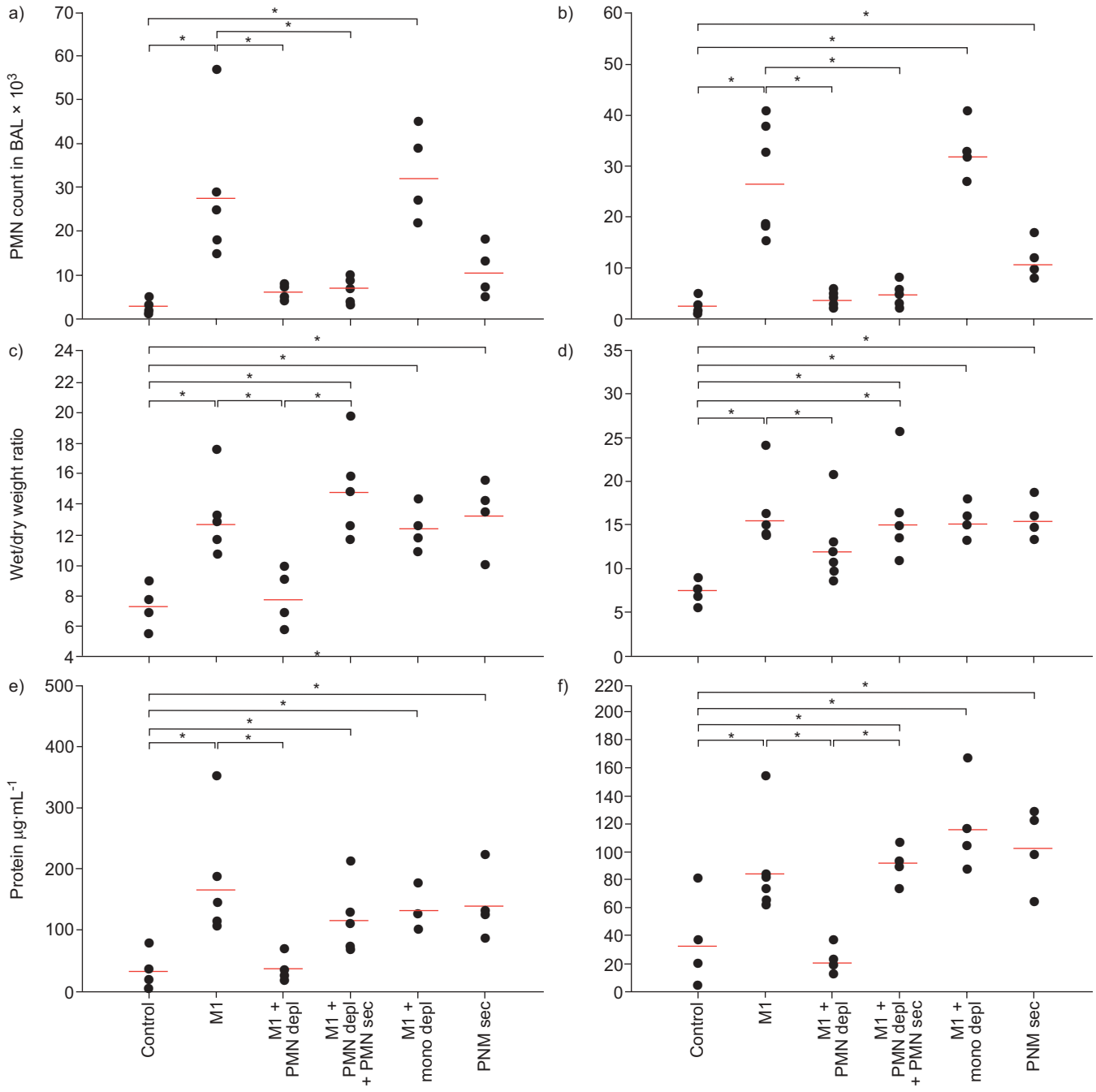

FIGURE 4. M1 protein-induced lung damage in mice was mediated by polymorphonuclear leukocyte (PMN) granule proteins: analysis of bronchoalveolar lavage (BAL) fluid and vascular leakage. Mice with intact white blood count were injected intravenously with buffer (control), M1 protein or PMN secretion (PMN sec). In separate experiments, mice were depleted of PMN (PMN depl) or monocytes (mono depl) prior to injection of M1 protein, in some experiments also combined with injection of PMN secretion. After 30 min (a, c, e) or 4 h (b, d, f) the mice were sacrificed. Individual data points are shown, along with mean values. a and b) Number of PMN in the cytospin of BAL fluid. c and d) Lung wet/dry weight ratio. e and f) Protein concentration in BAL fluid. *: $p<0.05$.

and, thus, are not supposed to be present in the PMN secretion. In line with this, tumour necrosis factor (TNF), interferon-γ, interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 could not be detected in the PMN secretion used (data not shown). Conversely, once the PMNs have extravasated, a second burst of transcriptional activity is launched, resulting in production of IL-1, TNF, IL-8 and MCP-1 [25].

DISCUSSION

S. pyogenes of the M1 serotype is commonly associated with large outbreaks of invasive streptococcal infections and the development of STSS. The fatal outcome of STSS is based mainly on the establishment of acute lung damage, characterised by severe oedema formation. It has been previously reported that M1 protein shed from the surface of *S. pyogenes*

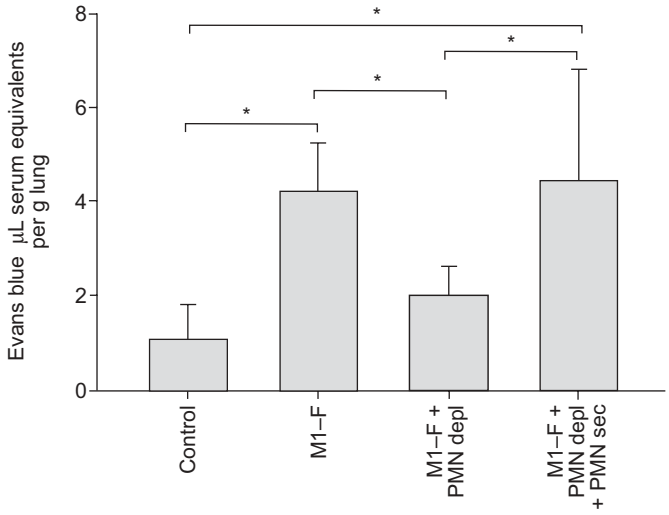

FIGURE 5. Injection of M1 protein–fibrinogen precipitates caused polymorphonuclear leukocyte (PMN)-dependent increase in vascular permeability in the lung. Complexes of M1 protein and fibrinogen (M1–F) were prepared *ex vivo* and injected into mice with intact white blood cells, neutropenic (PMN depl) mice or neutropenic mice injected with PMN secretion (PMN sec). Vascular protein leakage was assessed by Evans blue dye extravasation 30 min after injection of M1 protein–fibrinogen complexes. Data are presented as mean±SD. Each bar represents six mice. *: $p<0.05$.

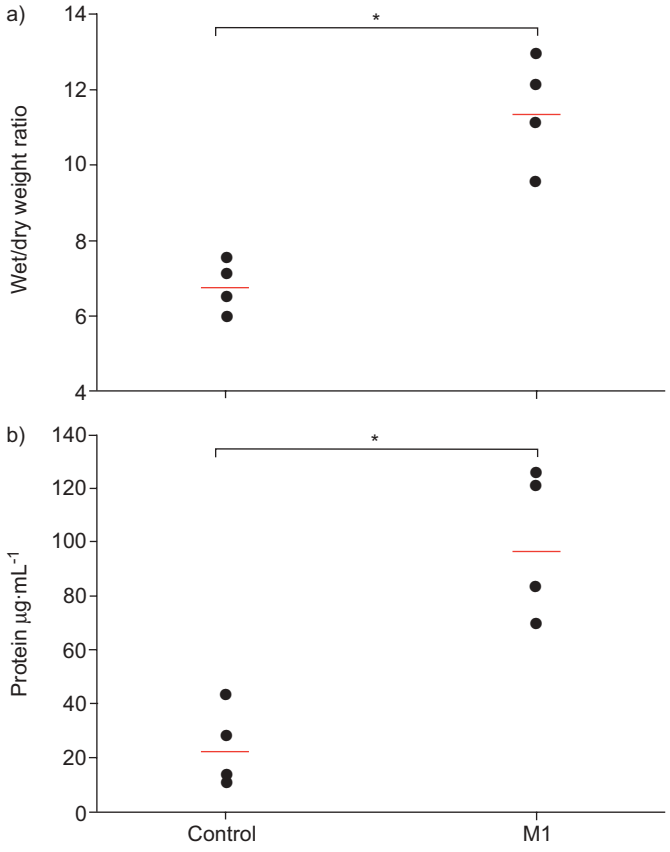

FIGURE 6. Murine polymorphonuclear leukocyte (PMN) secretion obtained from PMNs activated with M1 protein–fibrinogen precipitates induced lung damage, as determined by a) lung wet/dry weight ratio and b) protein concentration in bronchoalveolar lavage fluid. Supernatant from nonactivated murine PMNs or from murine PMNs activated with M1 protein–fibrinogen complexes was injected intravenously into mice. After 30 min the mice were sacrificed. Individual data points and means are shown. *: $p<0.05$.

forms complexes with fibrinogen that induce the activation of PMNs [5]. Shortly after, several other virulence mechanisms of M1 protein were reported, which may be involved in the pathogenesis of the acute lung damage in STSS. These comprise the activation of monocytes, T-cells and platelets, as well as the secretion of chemokines, cytokines and tissue factor [7–11]. The present study, however, points at an almost exclusive role of the intravascular activation of PMNs and the subsequent discharge of granule proteins in the onset of the M1 protein-induced lung oedema and lung damage.

Several granule proteins have been suggested to be critically involved in the progression of acute lung injury, among them proteases (elastase, gelatinase, cathepsin G and proteinase-3) and defensins [2]. The use of neutralising agents and gene-targeted mice has determined the individual contribution of these to the pathology of acute lung injury [26, 27]. The more general approach of the present study clearly demonstrates the significance of granule release in the pathophysiology of acute lung injury. Similarly interesting is the finding that the M1 protein–fibrinogen complexes activate PMNs intravascularly, so that a direct interaction between PMNs and the endothelium is not necessary for the development of the lung injury.

Intravenous injection of the tetrapeptide Gly-Pro-Arg-Pro ameliorates the M1 protein-induced lung damage [5], identifying PMN β_2-integrins as a possible target for interventions in fatal group A streptococcal infections. However, blockade of integrin function has been largely disappointing in trials in patients exhibiting various forms of inflammatory disease [28]. The present study puts neutrophil degranulation into perspective as a potential therapeutic target. Inhibition of neutrophil granule exocytosis may not only interfere with PMN extravasation but also influence vascular leakage and the second wave of inflammatory cell invasion [29], and thereby improve the outcome of a patient. Recently, a novel inhibitor of degranulation based on interference with myristoylated alanine-rich C kinase substrate has been developed that has promising results *in vitro* [30, 31], and further investigations are needed to prove the effectiveness *in vivo*. With respect to severe infections with *S. pyogenes*, it is noteworthy that leakage of plasma from the bloodstream into the surrounding tissue induces a life-threatening hypovolaemic hypotension combined with high morbidity and mortality. Therefore, the present study suggests that substances neutralising the effect of HBP or preventing PMN degranulation are an interesting target for drug development.

In conclusion, the present findings demonstrate that complexes formed by M1 protein shed from *Streptococcus pyogenes* and fibrinogen stimulate circulating polymorphonuclear leukocytes to degranulate. This response induces a rapid increase in lung vascular permeability, haemorrhage and deposition of fibrinogen precipitates, reflecting the entire picture of acute lung damage. Similar pathophysiological connections may exist where intravascular activation of polymorphonuclear leukocytes is associated with acute lung damage,

such as disseminated intravascular coagulation. The present data point to the powerful pernicious effect of polymorphonuclear leukocyte granule proteins in the early stages of acute lung injury, and may not only provide mechanistic insight but also stimulate therapeutic approaches that target polymorphonuclear leukocyte activation and degranulation rather than individual granule components.

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