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Mature CD11c$^+$ cells are enhanced in hypersensitivity pneumonitis

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ABSTRACT: The present study verified the hypothesis that enhanced maturation of antigen-presenting CD11c$^+$ cells could explain the viral-induced exacerbated immune response to *Saccharopolyspora rectivirgula* (SR), the main antigen responsible for farmer's lung, a classic form of hypersensitivity pneumonitis (HP).

Four groups of mice were studied: group 1 received intranasal instillations of saline; group 2 received instillations of SR for 12 weeks; group 3 received instillations of saline and a single infection with Sendai virus on week 3; and group 4 received instillations of SR for 12 weeks with a single administration of Sendai virus on week 3. On week 13, mice were sacrificed and bronchoalveolar lavage was performed. Lungs were harvested, digested with enzymes, and CD11c$^+$ cells were analysed in flow cytometry with anti-CD11c, anti-CD86 and anti-major histocompatibility complex class II markers. Immunofluorescence studies were also performed with the same cell surface markers.

Both flow cytometry and immunofluorescence results demonstrate that mature CD11c$^+$ cells are significantly enhanced in SR-challenged mice simultaneously infected with Sendai virus, compared with other groups. These CD11c$^+$ cells persist in the lung for 9 weeks after the virus infection.

Maturation of CD11c$^+$ cells could explain, at least in part, the virus-induced increased immune response to SR antigens in this model of HP, but mechanisms have still to be elucidated.

KEYWORDS: Antigen presentation, farmer's lung, mice, *Saccharopolyspora rectivirgula*, Sendai virus

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Hypersensitivity pneumonitis (HP) is an inflammatory lung disease caused by an exacerbated immune response to repeated inhalations of a variety of antigens. Causative agents, mostly organic in nature, include animal proteins, microbial products and plant particles [1]. The disease is characterised by a pulmonary infiltration and proliferation of activated lymphocytes [2]. In the bronchoalveolar lavage fluid (BALF) of patients with HP, the number and percentage of T-cells are increased to as high as 80% of the recovered cells [3]. Due to the wide range of causative antigens, HP can occur in many work or home environments. Farmer's lung, one of the most common forms of HP, is most frequently caused by *Saccharopolyspora rectivirgula* (SR), a thermophilic actinomycete found in poorly conserved and mouldy hay, straw or grain [4].

The pathogenesis of HP is complex and most of the mechanisms involved remain poorly understood. There is increasing evidence that promoting factors are necessary to develop the disease. Few individuals exposed to HP antigens develop clinical symptoms of the disease (estimated at three farmers in 1,000 for farmer's lung in Quebec, Canada), whereas >50% of Quebec dairy farmers develop a lymphocytic alveolitis but remain asymptomatic [5]. These persons seem to develop a tolerant response to HP antigens.

Many individuals suffering from HP report initial symptoms suggestive of respiratory viral infection at the onset of HP symptoms [6]. It has previously been demonstrated that mice infected with Sendai virus, a parainfluenza virus that causes a transient lung inflammation in mice, are more responsive to SR antigens. This exacerbated immune response persists for up to 30 weeks after the viral infection [7]. A possible mechanism by which a viral infection could enhance HP is by increasing the expression of the CD86 co-stimulatory molecule on antigen-presenting cells (APCs). The interaction of the CD86 co-stimulatory molecule on APCs with CD28 on T-cells is an essential step in the activation of T-lymphocytes, the cells that are so abundant in HP.

CD11c$^+$ cells, which include dendritic cells and possibly macrophages, are particularly effective

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regulators of immunity and potent APCs in the organism. In the lung, these cells have a high phagocytic activity and are specialised in antigen uptake. Following tissue damage or during a lung infection, dendritic cells and macrophages process antigens provoking their maturation, and present antigenic peptides to T-cells. Dendritic cells may also migrate to the lymphoid organs where they induce proliferation of antigen-specific T-cells, thereby initiating a protective immune response. For this reason, these cells are called professional APCs [8, 9]. Macrophages upregulate their B7 molecules (CD80/CD86) in response to binding to CD40 ligand on the T-cells, and increase their expression of major histocompatibility complex (MHC) class II molecules, thus allowing further activation of resting CD4 T-cells [10].

The aim of the present study was to verify the maturation state of $CD11c^+$ cells in the viral-induced exacerbated immune response to SR antigen in a well described HP mouse model. The possible enhanced expression of MHC class II and CD86 co-stimulatory molecules on $CD11^+$ cells would provoke a massive activation of T-lymphocytes leading to a pro-inflammatory environment and, consequently, trigger HP.

MATERIAL AND METHODS

Animals

Pathogen-free C57Bl/6 female mice were obtained from Charles River (St Constant, QC, Canada). Virus-infected animals were isolated and housed in an air-filtered laminar hood. The protocol was approved by the animal experimentation ethics committee of the Institut Universitaire de Cardiologie et de Pneumologie de l'Université Laval (Quebec City, QC, Canada) and all protocols were conducted according to the Helsinki recommendations.

Antigen and Sendai virus

Lyophilised SR antigen was reconstituted with pyrogen-free saline at 4 $mg \cdot mL^{-1}$ and stored at -80°C. Parainfluenza 1 (Sendai/52) virus was obtained from the American Type Culture Collection (Manassas, VA, USA).

Viral dose–response

In order to determine a sublethal dose that induces a recruitment of $CD11c^+$ cells in the lung, a viral dose–response was performed. Animals were anaesthetised with isoflurane and intranasally instilled with 50 μL containing 12.5, 25 or 50 haemagglutination units (HAU) of Sendai virus, or 50 μL of saline, and sacrificed 9 days later.

Induction of HP and viral infection

Animals were anaesthetised with isoflurane and instilled intranasally with 50 μL of the appropriate solution (saline, SR antigen at 4 $mg \cdot mL^{-1}$, Sendai virus at 12.5 HAU (optimal dose based on the dose–response described above), or SR antigen plus Sendai virus) as previously described [7]. Animals were sacrificed on week 13. See figure 1 for protocol clarification.

Bronchoalveolar lavage

After sacrifice, bronchoalveolar lavage (BAL) was performed using three aliquots of 1 mL PBS. Total cells were counted. Cytospin preparations were stained with the Hemacolor Stain Set (EM Diagnostic Systems, Middletown, VA, USA) and differential counts obtained.

Immunofluorescence and histopathological studies

Sections of lungs from the viral dose–response and the HP protocols were embedded in Optimal Cutting Temperature compound (Sakura Fineter, Torrance, CA, USA). One section was used for immunofluorescence studies. The first staining was performed with hamster anti-mouse CD11c (BD Biosciences, Mississauga, ON, Canada), combined with mouse anti-hamster biotin followed by incubation with streptavidin-fluorescein isothiocyanate (FITC). Double staining was achieved with either rat anti-mouse CD86 plus mouse anti-rat biotin or biotin-conjugated rat anti-mouse I-A/I-E (MHC class II) and streptavidin-AlexaFluor 350. Fluorescence was read by a Nikon Eclipse E600 microscope (Nikon Canada Inc., Mississauga, ON, Canada), with acquisition software SPOT advanced (version 3.1; Diagnostics Instruments Inc., Sterling Heights, MI, USA). Each picture was analysed blindly by three persons, compared and graded 0–3 for intensity and quantity of marked cells, where 0 was no fluorescence and 3 was most fluorescent picture. Remaining sections were stained with haematoxylin and eosin for histology. Inflammatory parameters in lung tissue (peribronchial, perivascular and parenchymal infiltration of inflammatory cells) were evaluated blindly by a senior lung pathologist. Total histology score was calculated and graded 0–4, where 0 was normal lung and 4 was diffuse maximal inflammation.

Lung enzymatic digestion

Tissues from each group of mice were cut into small fragments and suspended in RPMI 1640 containing collagenase (1 $mg \cdot mL^{-1}$; Sigma, St Louis, MO, USA) and DNAse (2 $\mu g \cdot mL^{-1}$; Sigma) for 1 h at 37°C. Cells were recovered by centrifugation and suspended at 10^8 cells per 0.2 mL in PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA.

Flow cytometric analysis and sorting of $CD11^+$ cells

Lung cells were incubated with phycoerythrin (PE)-conjugated anti-CD11c for 60 min at 4°C and double stained with FITC-conjugated anti-CD86 or FITC-conjugated anti-I-A/I-E MHC class II monoclonal antibodies. PE- and FITC- conjugated isotype mouse immunoglobulin were used as negative controls. All monoclonal antibodies were purchased from BD Biosciences. Cells were analysed in an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA).

Statistical analysis

Data are expressed as mean$\pm$SE values for graphical representation. For comparisons between group means, a one-way ANOVA was performed. We considered a p-value <0.05 as a significant difference between groups. For immunofluorescence studies, intraclass correlations were calculated to verify inter-reader reliability between evaluators.

RESULTS

Sendai virus dose–response

Mice were instilled with different Sendai virus concentrations (12.5, 25 or 50 HAU) or saline in order to determine a nonlethal but infectious dose of virus. Animals were sacrificed on day 9. A concentration of 12.5 HAU of Sendai virus was chosen because this dose induced a massive recruitment of cells in the lung. Moreover, this sublethal viral concentration induced alveolar cell necrosis and epithelial desquamation, characteristic of

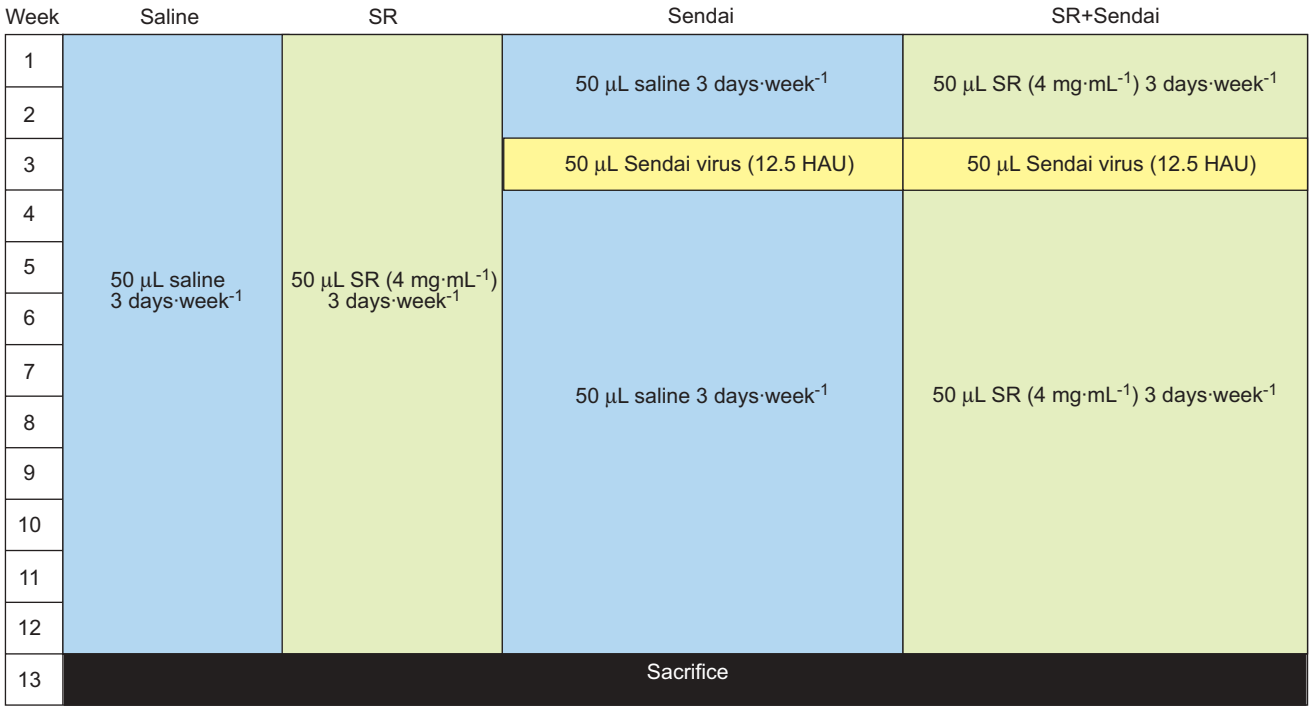

FIGURE 1. Study design. Four groups of mice were studied: group 1 received intranasal instillations of saline; group 2 received instillations of *Saccharopolyspora rectivirgula* (SR) for 12 weeks; group 3 received instillations of saline and a single infection with Sendai virus on week 3; and group 4 received instillations of SR for 12 weeks with a single administration of Sendai virus on week 3. Mice were sacrificed on week 13. HAU: haemagglutination units.

Sendai virus infection. The higher doses caused too severe damage, such as total epithelial destruction.

Bronchoalveolar lavage

Results of BAL total cell counts and differentials at 12 weeks are presented in figure 2. Low cell counts were obtained for the saline- and Sendai-instilled animals 9 weeks after infection. Repeated exposures to SR antigen induced a moderate increase of BAL cells that persisted up to the end of the 12 weeks studied. Most importantly, repeated SR challenges with one simultaneous Sendai virus infection at week 3 resulted in a persisting exacerbated cellular response to SR antigen. All BAL cell populations were moderately increased by repeated exposures to SR antigens but the increases were much higher in animals which were previously infected with Sendai virus. It is important to note that this enhanced immune response was still present long after the transient Sendai virus effect had waned, as shown by the control group of mice infected with the Sendai virus.

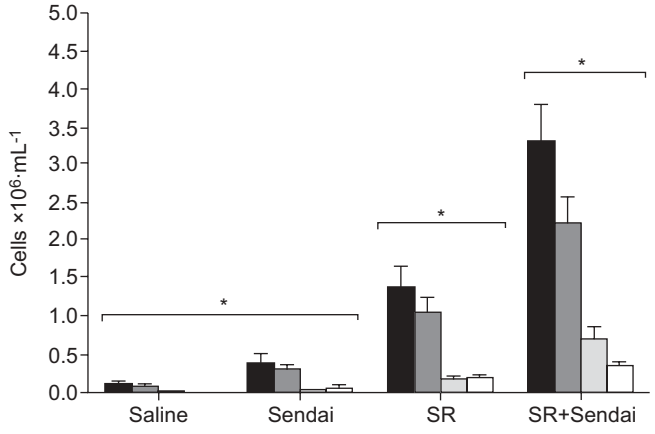

FIGURE 2. Total (■) and differential cell counts in bronchoalveolar lavage (BAL) cells, 9 weeks after viral infection. ■: macrophages; ■: lymphocytes; □: neutrophils. Numbers of total cells and subpopulations are expressed per millilitre of recovered BAL fluid (mean±SEM) for the various groups. A significant difference was observed between the *Saccharopolyspora rectivirgula* (SR)+Sendai group and the other groups (total cells: $p<0.003$; macrophages: $p<0.006$; lymphocytes: $p<0.005$; neutrophils: $p<0.03$). Eight mice per group. *: $p<0.05$ *versus* other bracketed groups.

Flow cytometry analysis of lung CD11c$^+$ cells

Similar to the cell populations recovered from BALF, lung CD11c$^+$ cells were markedly increased in mice that were sensitised to SR antigens and infected concomitantly with Sendai virus, compared with the other groups. Flow cytometry analysis showed that saline- and Sendai-instilled animals had similar levels of lung CD11c$^+$ cells, 9 weeks after the Sendai virus instillation in the appropriate group (fig. 3). Mice exposed to repeated SR challenges had an increased proportion of CD11c$^+$ cells compared with the saline and Sendai group. Repeated exposures to SR antigen with one instillation of Sendai virus resulted in a strong influx of lung CD11c$^+$ cells that persisted at 9 weeks after the virus infection. Moreover, as shown in figure 3, the expression of maturation markers was strongly increased on lung CD11c$^+$ cells of mice of the SR+Sendai group.

Immunofluorescence studies

Immunofluorescence studies were performed in order to further confirm the enhancement of CD86 and MHC class II

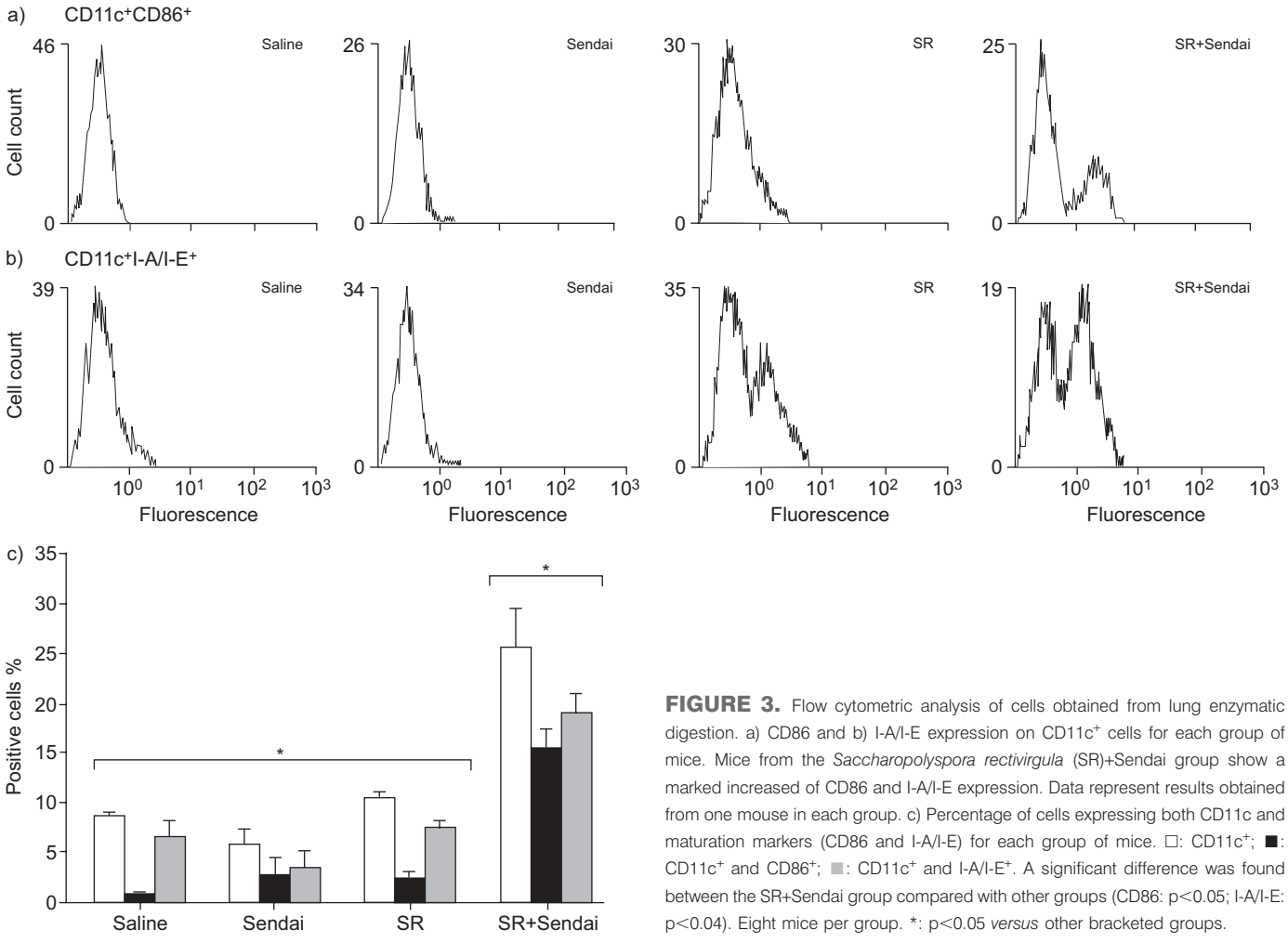

FIGURE 3. Flow cytometric analysis of cells obtained from lung enzymatic digestion. a) CD86 and b) I-A/I-E expression on $CD11c^+$ cells for each group of mice. Mice from the *Saccharopolyspora rectivirgula* (SR)+Sendai group show a marked increased of CD86 and I-A/I-E expression. Data represent results obtained from one mouse in each group. c) Percentage of cells expressing both CD11c and maturation markers (CD86 and I-A/I-E) for each group of mice. □: $CD11c^+$; ■: $CD11c^+$ and $CD86^+$; ■: $CD11c^+$ and I-A/I-E^+. A significant difference was found between the SR+Sendai group compared with other groups (CD86: $p<0.05$; I-A/I-E: $p<0.04$). Eight mice per group. *: $p<0.05$ *versus* other bracketed groups.

expression on $CD11c^+$ cells in lung tissues in this model of HP. Lung sections were first labelled with anti-CD11c FITC and double stained with anti-CD86 or anti-I-A/I-E (MHC class II) AlexaFluor. Slides were then evaluated blindly by three persons and intensity and quantity of marked cells were scored 0–3 (fig. 4). Lung cells from mice of the saline, Sendai and SR groups showed few with CD11c expression, whereas those of the SR+Sendai group had a marked increase of double-stained cells ($CD11c^+/CD86^+$ and $CD11c^+/I\text{-}A/I\text{-}E^+$).

Histopathology

Following haematoxylin and eosin staining, inflammatory parameters in lung tissue (peribronchial, perivascular and parenchymal infiltration of inflammatory cells) were evaluated blindly by a senior lung pathologist. The total histology score was calculated and graded 0–4, where 0 was normal lung and 4 was diffuse maximal inflammation (fig. 5). Both the saline- and the Sendai-instilled mice had normal lungs. Mice challenged with SR antigens showed a peribronchiolar and perivascular hyperplasia and important lung injuries, but damage was more severe in mice exposed to SR antigens and infected with the Sendai virus.

DISCUSSION

The current study confirms our previous findings that, in mice, a viral infection leads to an exacerbated immune response to SR, a HP antigen [7]. Most importantly, our results suggest a role for $CD11c^+$ cells in this lung response to inhaled antigen exposure. The migration of $CD11c^+$ cells, as well as the CD86 and MHC class II expression on these cells, were increased in mice repeatedly exposed to SR antigen and concomitantly infected once with Sendai virus. Animals from the control group of mice, infected with Sendai virus but not sensitised with SR antigen, fully recovered from the viral infection. The mechanisms involved in this $CD11c^+$ cell recruitment and maturation have still to be elucidated. Contact with other cell types and cytokine/chemokine signals should be essential in this migration and maturation of $CD11c^+$ cells in this setting. Kinetic studies could also be performed in order to define the cell population mobilised by the viral infection and to verify whether the final mature $CD11c^+$ cell population is the same as that which was present at the beginning of the protocol.

In this study, we used three markers to identify and characterise $CD11c^+$ cells. CD11c is a well described dendritic cell marker but it can also be upregulated on macrophages. However, studies show that mouse dendritic cells express

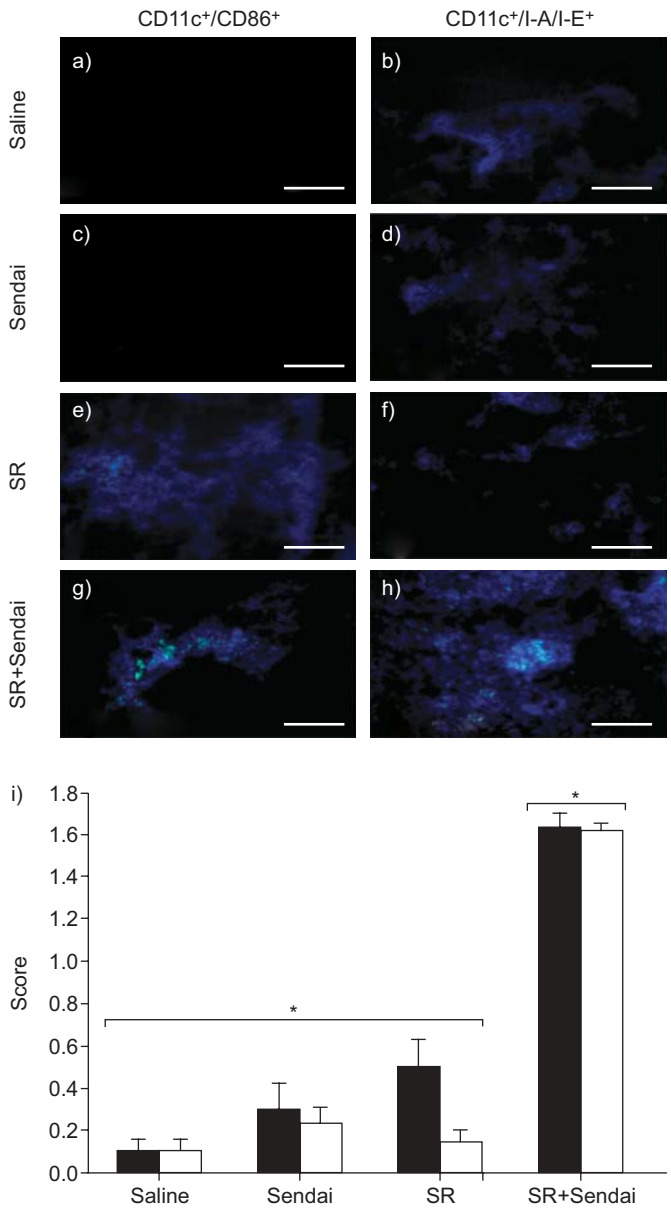

FIGURE 4. Immunofluorescence studies of lung sections from each group of mice. Tissue sections were marked with anti-CD11c fluorescein isothiocyanate (green) and double stained with a, c, e and g) anti-CD86 or b, d, f and h) anti-I-A/I-E AlexaFluor (blue). a and b) Saline; c and d) Sendai; e and f) *Saccharopolyspora rectivirgula* (SR); g and h) SR and Sendai. Scale bars=100 μm. i) Slides were blindly evaluated by three persons and intensity and quantity of marked cells were scored (graded on an arbitrary scale from 0 to 3; 0: no fluorescence; 1: low; 2: medium; 3: high). ■: $CD11c^+$ and $CD86^+$; □: $CD11c^+$ and I-A/I-E^+. Intraclass correlation coefficients showed that evaluators agreed for each marker (CD11c: 0.8913; CD86: 0.9469; I/A-I/E: 0.9519; $p<0.02$; eight mice per group). *: $p<0.05$ *versus* other bracketed groups.

MHC class II (I-A/I-E) in high density, whereas mouse macrophages have low-density MHC class II [11]. Some studies only used CD11c marker associated with MHC class II marker to define dendritic cells [12, 13]. Our results show that the majority of the $CD11c^+$ cells also express the I-A/I-E (MHC class II) marker (fig. 3a), and could be considered as dendritic cells. However, although macrophages typically do not express MHC class II, those isolated from lung interstitium could express class II variably. Hence, cell isolation by fluorescence-activated cell sorting (FACS) will be necessary to confirm if these $CD11c^+$ cells are dendritic cells or macrophages. Additional markers like CD68 have to be used to differentiate between dendritic cells and macrophages. Maturation of dendritic cells and macrophages is associated with an upregulation of the co-stimulatory molecule CD86 [14, 15]. We used this marker to determine the maturation state of cells. Moreover, because plasmacytoid dendritic cells (CD11c low) have a key role in viral immunity, other studies must be performed in order to verify whether these cells could have a role in HP [16].

The maturation of CD11c APCs could have a major role in the pathophysiology of HP. These cells are often the first cells to encounter antigens. This contact triggers their maturation. Mature APCs process and present antigen to T-cells. Activated T-cells are the major cells in HP. The maturation of $CD11c^+$ cells could explain, in part, the intense recruitment of T-cells in lungs of mice from the SR+Sendai group and the following inflammation compared with other groups.

Animal models are helpful in understanding events occurring in human diseases. However, even if they are similar in many aspects, physiopathological processes can differ in mice and humans. Our model used SR-sensitive mice, which is important considering that the objective was to study the exacerbated immune response to this antigen in a human disease. The choice of a virus infection was based on findings that HP patients often complain of flu-like symptoms and that viral antigens are more expressed in lung tissue of HP patients than in normal subjects [6]. Given that Sendai virus is a common rodent parainfluenza virus similar to human influenza virus [17, 18], we felt that this infectious agent was appropriate to answer our questions. Moreover, this model has previously been extensively used and it has previously been shown that a viral infection enhances the lung response to SR antigen well beyond the transient viral infection [7].

The enhanced immune response is not specific to Sendai virus or to a viral infection. Studies by GUDMUNDSSON *et al.* [19] show similar results using SR antigen and respiratory syncytial virus. Like Sendai virus, respiratory syncytial virus is a negative-sense, single-stranded (ss)RNA virus of the family Paramyxoviridae. A study by FOGELMARK *et al.* [20] demonstrated that exposure to a combination of endotoxin and β(1,3)-D-glucan provokes a histology resembling HP with alveolar infiltrates and early granulomas. In the future, ultraviolet light-inactivated Sendai virus should be used as a control for Sendai virus infection. This would allow for the mimic of ssRNA and antigen administered, whereas saline fails to provide these APC stimulants.

The maturation and activation of $CD11c^+$ cells by Sendai virus is controversial. Some authors claim that viruses suppress dendritic cell function. A study by YONEMITSU *et al.* [21] demonstrated that *in vitro* infection of immature dendritic cells with Sendai virus provokes spontaneous maturation and activation. However, ARMEANU *et al.* [22], in a similar study, showed that dendritic cells exposed to lipopolysaccharide

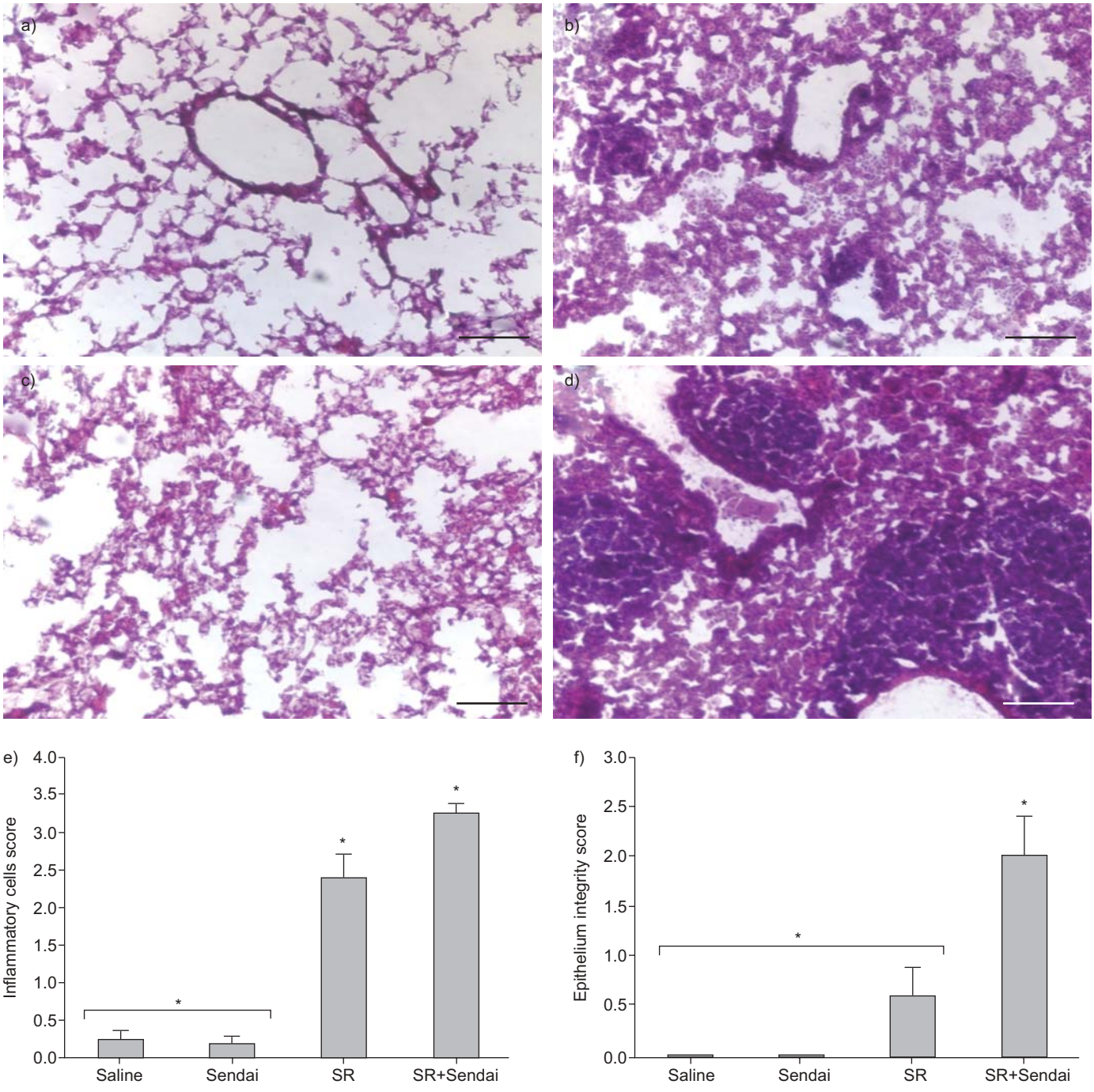

FIGURE 5. Histopathology results of mice instilled with a) saline, b) *Saccharopolyspora rectivirgula* (SR), c) Sendai and d) SR+Sendai. SR+Sendai treatment induced a severe peribronchiolar and perivascular hyperplasia as well as important lung damage compared with SR-treated mice, whereas saline- and Sendai-treated mice had normal lungs. Scale bars=50 μm. e) Results are expressed as the total histological score (graded on an arbitrary scale from 0 to 4; 0: no inflammatory cells; 1: <10%; 2: 10–25%; 3: 25–50%; 4: >50%), which was significantly increased in SR+Sendai-treated mice (total score of 3.25±0.13) compared with other mice (saline: 0.25±0.1; Sendai: 0.18±0.1; SR: 2.4±0.3) ($p<0.02$). f) Epithelial integrity was also scored from 0 to 3. 0: no epithelial damage; 1: low damage; 2: moderate damage; 3: severe epithelial damage. SR+Sendai mice showed high epithelial damage compared with other groups ($p<0.01$; eight mice per group). *: $p<0.05$ *versus* other bracketed groups.

following a virus infection have severely impaired allostimulatory activity. In our study, $CD11c^+$ cells showed an increased expression of CD86 and MHC class II molecules. Functional tests have to be performed in order to confirm whether allostimulatory activity is impaired. The fact that we used an *in vivo* model could explain the maturation and the possible activation of $CD11c^+$ cells. Some molecules, like interferon, which provoke maturation and activation of $CD11c^+$ cells, could have been secreted by other cells, a situation that seems to be impossible in an *in vitro* setting. To be effective in the

viral immunity, macrophages must avoid their own virus-induced death. TYNER *et al.* [23] demonstrated that the interaction between CC chemokine ligand 5 and CC chemokine receptor 5 provides antiapoptotic signals for macrophage survival during a Sendai virus infection. Moreover, macrophages could contribute to airway hyperresponsiveness by decreasing M_2 receptor function after a Sendai virus infection [24]. Hence, macrophages seem to be effective during a Sendai virus infection but functional tests will also have to be performed to confirm this.

An intriguing observation is that, although the viral infection does cause a recruitment in $CD11c^+$ cells that express CD86 and MHC class II during the acute phase, these cells rapidly return to normal values as shown by our results at week 9 in the group of mice infected with the virus only. These findings parallel those seen in a study by STUMBLES *et al.* [25], where mature dendritic cell recruitment into resting and inflamed airways was observed in a rat model of Sendai virus infection. However, when the virus infection was introduced simultaneously with SR antigen and when the antigen administration was continued, the mature dendritic cells persisted up to sacrifice at 9 weeks. A possible explanation is that, when the SR antigen is given during the Sendai infection, the combination of disrupted epithelial barrier and the presence of mature dendritic cells results in an increase antigen presentation to lymphocytes. Once this is produced, the resulting increase in the immune response is sufficient to self-perpetuate the antigenic aggression and continued increase in antigen presentation by the persisting mature dendritic cells. This ongoing inflammation could prevent tissue repair which, in turn, could exacerbate the inflammation. Given that farm workers are continuously exposed to the antigen, the hypersensitisation could persist until contact avoidance. In our model, one could equate the mild response to the SR antigen to that of asymptomatic exposed individuals and the enhanced response induced by the viral infection to active HP.

Recently, many studies have demonstrated that dendritic cells are able to instruct T-cells' response, to induce tolerance rather than immunity [26]. These tolerogenic dendritic cells are able to drive the differentiation of T-regulatory cells, a T-cell subset with suppressive properties. T-regulatory cells have the capacity to actively suppress the proliferation of naive $CD4^+$ T-cells by secreting interleukin-10 and transforming growth factor-β, two effective immunosuppressive cytokines [27]. These cytokines can, in turn, prevent dendritic cell maturation [28]. An increased number of T-regulatory cells could explain the tolerant response observed in mice exposed to SR antigen, as well as in asymptomatic exposed subjects. Further studies are needed to elucidate this tolerance mechanism.

Conclusion

This study clearly shows that mature $CD11c^+$ cells are enhanced in a mouse model of HP. This maturation enhances the antigen-presenting function of $CD11c^+$ cells, which could lead to increased T-cell proliferation and survival. The resulting enhanced immune response maintains inflammation, which prevents tissue repair, which, in turn, allows easier access of the antigen for $CD11c^+$ cells. Further studies are needed to explain the potential tolerogenic state of $CD11c^+$ cells during SR exposure, as well as immune mechanisms involved in the $CD11c^+$ cell maturation process during HP. FACS cell isolation studies will be needed to discriminate between dendritic cells and macrophages. Moreover, dendritic cell- or macrophage-depleted mice could be an interesting model to document whether or not these cells are essential in HP pathogenesis.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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