ERJ Express. **Published on March 12, 2009 as doi: 10.1183/09031936.00140908**

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

Authors: M. Girard, E. Israël-Assayag, and Y. Cormier.

Affiliation: Unité de Recherche, Centre de Pneumologie, Institut Universitaire de Cardiologie et de Pneumologie de l'Université Laval, Hôpital Laval, Quebec City, Québec, Canada, G1V 4G5

Short title: CD11c$^+$ cells in hypersensitivity pneumonitis

Contact information:

Dr Yvon Cormier

Hôpital Laval

2725, chemin Sainte-Foy

Quebec City, Québec

Canada

G1V 4G5

Tel: 418 656 4747

Fax: 418 656 4762

e-mail address: Yvon.Cormier@med.ulaval.ca

This work was funded by Canadian Institutes of Health Research (CIHR)

Abstract

The present study verified the hypothesis that an 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, instillations of SR during 12 weeks; Group 3, instillations of saline and a single infection with Sendai virus on week three; and Group 4, instillations of SR during 12 weeks with a single administration of Sendai virus on week three. On week twelve, mice were sacrificed and bronchoalveolar lavages (BAL) were performed. Lungs were harvested, digested with enzymes, and CD11c$^+$ cells were analyzed in flow cytometry with anti-CD11c, -CD86 and -MHC 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 to 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 still to be elucidated.

Key words: Antigen presentation, farmer's lung, mice, Saccharopolyspora rectivirgula, Sendai virus

Introduction

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 characterized 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]. Because of the wide range of causative antigens, HP can occur in many work or home environments. Farmer's lung (FL), 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 are increasing evidences 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 FL in Quebec) whereas more than 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]. We have previously 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 (APC). The interaction of the CD86 co-stimulatory molecule on APC with CD28 on T cells is an essential step in the activation of T lymphocytes, cells which are so abundant in HP.

CD11c$^+$ cells, which include dendritic cells and possibly macrophages, are particularly effective regulators of immunity and potent APC in the organism. In the lung, these cells have a high phagocytic activity and are specialized 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 APC [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 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, Canada). Virus-infected animals were isolated and housed in air filtered laminar hood. The protocol was approved by our institution animal experimentation ethics committee and all protocols were conducted according to the Helsinki recommendations.

Antigen and Sendai virus

Lyophilized SR antigen was reconstituted with pyrogen-free saline at 4 mg/ml and stored at -80^o C. Parainfluenza 1 (Sendai/52) virus was obtained from the American Type Culture Collection (Manassas, USA).

Viral dose-response

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

Induction of HP and Viral Infection (see Table 1 for protocol clarification)

Animals were anesthetised with isoflurane and instilled intranasally with 50 µl of the appropriate solution (saline, SR antigen; 4mg/ml, Sendai virus; 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 12.

Bronchoalveolar Lavage

After sacrifice, BAL was performed by using three aliquots of 1 ml phosphate-buffered saline (PBS). Total cells were counted. Cytospin preparations were stained with Hemacolor Stain Set (EM Diagnostic Systems, Middletown, USA), and differential counts obtained.

Immunofluorescence and histopathologic studies

Sections of lungs from the viral dose response and the HP protocols were embedded in Optimal Cutting Temperature (OCT) compound. One section was used for immunofluorescence studies. The first staining was performed with hamster anti-mouse CD11c (BD Biosciences; Mississauga, Canada), combined with mouse anti-hamster biotin followed by incubation with Streptavidin-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 with acquisition software SPOT advanced (version 3.1). Each picture was analysed blindly by three persons, compared and graded from 0 to 3 for intensity and quantity of marked cells, where 0 = no fluorescence and 3 = most fluorescent picture. Remaining sections were stained with hematoxylin and eosin (H&E) 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 from 0–4, where 0 = normal lung and 4 = diffuse maximal inflammation.

Lung enzymatic digestion

Tissues from each groups of mice were cut into small fragments and suspended in RPMI 1640 containing collagenase (1 mg/ml; Sigma, St-Louis, USA) and DNAse (2 µg/ml; Sigma, St-Louis, USA) for 1 hour at 37^o C. Cells were recovered by centrifugation and suspended at 10^8 cells / 0.2 ml in PBS supplemented with 0.5% BSA and 2mM EDTA.

Flow Cytometric Analysis and Sorting of CD11$^+$ cells

Lung cells were incubated with PE-conjugated anti-CD11c for 60 minutes at 4^{o}C and double stained with FITC-conjugated anti-CD86 or FITC-conjugated anti-I-A/I-E MHC class II mAbs. PE- and FITC- conjugated isotype mouse Ig were used as negative controls. All mAbs were purchased from BD Biosciences (Mississauga, Canada). Cells were analysed in an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, USA).

Statistical analysis

Data are expressed as mean values ± S.E. for graphical representation. For comparisons between group means, a one-way analysis of variance (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 to determine a non-lethal 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 lung. Moreover, this sub-lethal viral concentration induced alveolar cells necrosis and epithelial desquamation characteristic of Sendai virus infection. The other doses caused too severe damages like total epithelial destruction.

Bronchoalveolar Lavage

Results of BAL total cell counts and differentials at twelve weeks are presented in Figure 1. Low cell counts were obtained for the saline- and Sendai-instilled animals 9 weeks post 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

Flow cytometry analysis of lung CD11c$^+$ cells

Similarly to the cell populations recovered from BALF, lung CD11c$^+$ cells were markedly increased in mice that were sensitized to SR antigens and infected concomitantly with Sendai virus compared to the other groups. Flow cytometry analysis showed that Saline- and Sendai-instilled animals had similar levels of lung CD11c$^+$ cells, nine weeks after the Sendai virus instillation in the appropriate group (Figure 2). Mice exposed to repeated SR challenges had an increased proportion of CD11c$^+$ cells compared to 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 2, the expression of maturation markers was strongly increased on lung CD11c$^+$ cells of mice of the SR + Sendai group.

Immunofluorescence studies

Immunofluorescence studies were done to further confirm the enhancement of CD86 and CMH class II 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 where scored from 0 to 3 (Figure 3). Lung cells from mice of the saline, Sendai and SR groups showed few CD11c expression whereas those of the SR +Sendai group had a marked increase of double stained cells (CD11c$^+$/CD86$^+$, CD11c$^+$/ I-A/I-E$^+$ (MHC class II).

Histopathology

Following a Haematoxylin & Eosin staining, 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 from 0–4, where 0 = normal lung and 4 = diffuse maximal inflammation (Figure 4). 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 that were not sensitized with SR antigen have fully recovered from the viral infection. The mechanisms involved in this CD11c$^+$ cells recruitment and maturation still to be elucidated. Contact with other cell types and cytokines/chemokines signal 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 mobilized by the viral infection and to verify if the final mature CD11c$^+$ cell population is the same as that which was present at the beginning protocol.

In this study, we used three markers to identify and characterize CD11c$^+$ cells. CD11c is a well described DC markers but it can also be upregulated on macrophages. However, studies show that mice DCs express major histocompatibility class II (I-A/I-E) in high density whereas mice macrophages have low-density MHC class II [11]. Some studies only used CD11c marker associated with MHC class II marker to define DC [12, 13]. Our results show that the majority of the CD11c$^+$ cells also express the I-A/I-E (MHC class II) marker (Figure 2a), and could be considered as DCs. However, although macrophages typically are do not express MHC class II, those isolate from lung interstitium could express class II variably. Hence, cells isolation by 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 costimulatory 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 done to verify if these cells could have a role in HP [16].

The maturation of CD11c antigen-presenting cells (APC) could have a major role in the pathophysiology of hypersensitivity pneumonitis. These cells are often the first cells to encounter antigens. This contact triggers their maturation. Mature APC 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 lung of mice from the SR + Sendai group and the following inflammation compared to 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 we have previously 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 nor to a viral infection. Studies by Gudmundsson et al. show similar results using SR antigen and respiratory syncytial virus (RSV) [19]. Like Sendai virus, RSV is a negative-sense, single-stranded RNA (ssRNA) virus of the family Paramyxoviridae. A study by Fogelmark et al. demonstrates that exposure to a combination of endotoxin and beta (1-3) -D-glucan provokes a histology resembling HP with alveolar infiltrates and early granulomas [20]. In the future, ultraviolet light inactivated Sendai virus should be used as a control for Sendai virus infection. This allows 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 virus suppress dendritic cells function. A study of Yonomitsu et al.

demonstrates that *in vitro* infection of immature dendritic cells with Sendai virus provokes spontaneous maturation and activation [21]. However, Armeanu et al., in a similar study, showed that dendritic cells exposed to LPS following a virus infection have severely impaired allostimulatory activity [22]. In our study, CD11c$^+$ cells show an increased expression of CD86 and MHC class II molecules. Function tests have to be performed to confirm if 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 seem to be impossible in an *in vitro* setting. To be effective in the viral immunity, macrophage must avoid its own virus-induced death. Tyner et al. demonstrate that CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during a Sendai virus infection [23]. 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 function test will also have to be performed to confirm this affirmation.

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 of Stumbles et al. where mature DCs recruitment into resting and inflamed airways was observed in a rat model of Sendai virus infection [25]. However, when the virus infection was produced simultaneously with SR antigen and when the antigen administration was continued the mature DCs 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 DCs 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 DCs. 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 hypersensitization 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 DCs are able to instruct T cells response to induce tolerance rather than immunity [26]. These tolerogenic DCs are able to drive the differentiation of T regulatory (T reg) cells, a T cells subset with suppressive properties. T reg cells have the capacity to actively suppress the proliferation of naïve CD4$^+$ T cells by secreting IL-10 and transforming growth factor beta (TGF-β), two effective immunosuppressive cytokines [27]. These cytokines can, in turn prevent DCs maturation [28]. An increased number of T reg cells could explain the tolerant response observed in mice exposed to SR antigen as well as 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 antigen-presenting function of CD11c$^+$ cells which could lead to an increased T cells 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$^+$ cells maturation process during HP. FACS cells isolation studies will be needed to discriminate between dendritic cells and macrophages. Moreover, studies in dendritic cells- or macrophages-depleted mice could be an interesting model to document whether these cells are essential or not in HP pathogenesis.

Grants

Authors thank CIHR and partner institutes for financial support. Melissa Girard awarded a scholarship from the PHARE Training Program of the CIHR.

References

1. Cormier Y, Israël-Assayag E. Pathogenesis of hypersensitivity pneumonitis. In: *Textbook of respiratory cell and molecular biology*, edited by Martin Dunitz Ltd, 2002.

2. Suga M, Yamasaki H, Nakagawa K, Kohrogi H, Ando M. Mechanisms accounting for granulomatous responses in hypersensitivity pneumonitis. *Sarcoidosis Vasc Diffuse Lung Dis* 1997; 14: 131-138.

3. Semenzato G, Chilosi M, Ossi E, Trentin L, Pizzolo G, Cipriani A, Agostini C, Zambello R, Marcer G, Gasparotto G. Bronchoalveolar lavage and lung histology : Comparative analysis of inflammatory and immunocompetent cells in patients with sarcoidosis and hypersensitivity pneumonitis. *Am Rev Respir Dis* 1985; 132: 400-404.

4. Pepys J, Jenkins PA, Festenstein GN, Gregory PH, Lacey ME, Skinner FA. Farmer's lung: thermophilic actinomycetes as a source of "farmer's lung hay" antigen. *Lancet* 1963; 2: 607-611.

5. Cormier Y, Bélanger J, Beaudoin J, Laviolette M, Beaudoin R, Hébert J. Abnormal bronchoalveolar lavage in asymptomatic dairy farmers : a study of lymphocytes. *Am Rev Respir Dis* 1984; 130: 1046-1049.

6. Dakhama A, Hegele RG, Laflamme G, Israel-Assayag E, Cormier Y. Common respiratory viruses in lower airways of patients with acute hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 1999; 159: 1316-1322.

7. Cormier Y, Tremblay GM, Fournier M, Israël-Assayag E. Long-term viral enhancement of lung response to *Saccharopolyspora rectivirgula*. *Am J Respir Crit Care Med* 1994; 149:490-494.

8. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; 392: 245-252.

9. Lambrecht BN. Allergen uptake and presentation by dendritic cells. *Curr Opin All Clin Immunol* 2001; 1: 51-59.

10. Harris NL, Ronchese F. The role of B7 costimulation in T-cells immunity. *Immunol Cell Biol* 1999; 77: 304-311.

11. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; 9: 271-296.

12. Jae-Hoon Choi, Yoonkyung Do, Cheolho Cheong, Hyein Koh, Silvia B. Boscardin, Yong-Seok Oh, Leonia Bozzacco, Christine Trumpfheller, Chae Gyu Park, Ralph M. Steinman. Identification of antigen-presenting dendritic cells in mouse aorta and cardiac valves. *J Exp Med* 2009, in press.

13. Osterholzer JJ, Surana R, Milam JE, Montano GT, Chen GH, Sonstein J, Curtis JL, Huffnagle GB, Toews GB, Olszewski MA. Cryptococcal Urease Promotes the Accumulation of Immature Dendritic Cells and a Non-Protective T2 Immune Response within the Lung. *Am J Pathol* 2009, in press.

14. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; 18: 767-811.

15. Israël-Assayag E, Dakhama A, Lavigne S, Laviolette M, Cormier Y. Expression of costimulatory molecules on alveolar macrophages in hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 1999; 159:1830-1834.

16. Park SS, Ehlenback SJ, Grayson MH. Lung dendritic cells and IgE: the link between virus and atopy. *Future Microbiol* 2008; 3: 241-245.

17. Chanock RM, McIntosh K. Parainfluenza viruses. In: *Virology*, edited by Fields BN, Knipe DM : Raven Press, 1990.

18. Kido H, Murakami M, Oba K, Chen Y, Towatari T. Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. *Mol cells* 1999; 9: 235-244.

19. Gudmundsson G, Monick MM, Hunninghake GW. Viral infection modulates expression of hypersensitivity pneumonitis. *J Immunol* 1999; 162: 7397-7401.

20. Fogelmark B, Sjostrand M, Rylander R. Pulmonary inflammation induced by repeated inhalations of beta(1-3)-D-glucan and endotoxin. *Int J Exp Pathol* 1994; 75: 85-90.

21. Yonemitsu Y, Ueda Y, Kinoh H, Hasegawa M. Immunostimulatory virotherapy using recombinant Sendai virus as a new cancer therapeutic regimen. *Front Biosci* 2008; 13: 4953-4959.

22. Armeanu S, Bitzer M, Smirnow I, Bossow S, Appel S, Ugerechts G, Bernloher C, Neubert WJ, Lauer UM, Brossart P. Severe impairment of dendritic cells allostimulatory activity by Sendai virus vector is overcome by matrix protein gene deletion. *J Immunol* 2005; 175: 4971-4980.

23. Tyner JW, Uchida O, Kajiwara N, Kim EY, Patel AC, O'Sullivan MP, Walter MJ, Schwendener RA, Cook DN, Danoff TM, Holtzman MJ. CCL5-CCR5 interaction provides antiapoptotic signals for macrophages survival during viral infection. *Nat Med* 2005; 11: 1180-1187.

24. Lee AM, Fryer AD, van Rooijen N, Jocoby DB. Role of macrophages in virus-induced airway hyperresponsiveness and neuronal M2 muscarinic receptor dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L1255-L1259.

25. Stumbles PA, Strickland DH, Pimm CL, Proksch SF, Marsh AM, McWilliam AS, Bosco A, Tobagus I, Thomas JA, Napoli S, Proudfoot AE, Wells TN, Holt PG. Regulation of dendritic cell recruitment into resting and inflamed airway

epithelium: Use of alternative chemokine receptors as a function of inducing stimulus. *J Immunol* 2001; 167: 228-234.

26. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* 2005; 105: 1162-1169.

27. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004; 10: 801-805.

28. Brown RD, Pope B, Murray A, Esdale W, Sze DM, Gibson J, Ho PJ, Hart D, Joshua D. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. *Blood* 2001; 98: 2992-2998.

Figures legends

Figure 1: Total and differential cell counts in BAL cells, 9 weeks post viral infection. Number of total cells and subpopulations are expressed per milliliter of recovered BALF (mean ± SEM) for the various groups. A significant difference was observed between SR + Sendai group versus other groups: Total cells ($p < 0.003$); Macrophages ($p < 0.006$); Lymphocytes ($p < 0.005$) ; Neutrophils ($p < 0.03$). n = 8 mice per group.

Figure 1

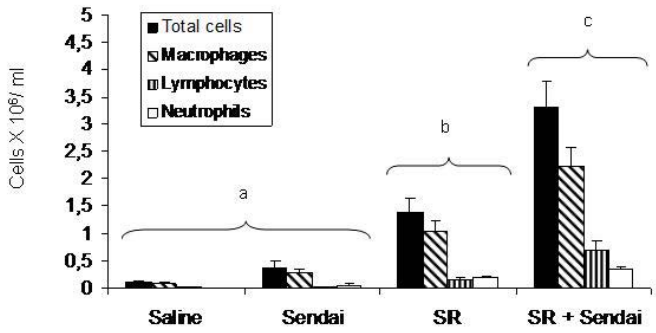


Figure 2: Flow cytometric analysis of cells obtained from lung enzymatic digestion. a) Percentage of cells expressing both CD11c and maturation markers (CD86 and I-A/I-E) for each group of mice. A significant difference was found between the SR + Sendai compared to other groups (CD86: $p < 0.05$; I-A/I-E: $p <$

0.04). n = 8 mice per groups. **b)** CD86 and I-A/I-E expression on CD11c+ cells for each group of mice. Mice from the SR + Sendai group show a marked increased of the CD86 and I-A/I-E expression. Data represent results obtain from 1 mouse in each group. n = 8 mice per groups.

Figure 2

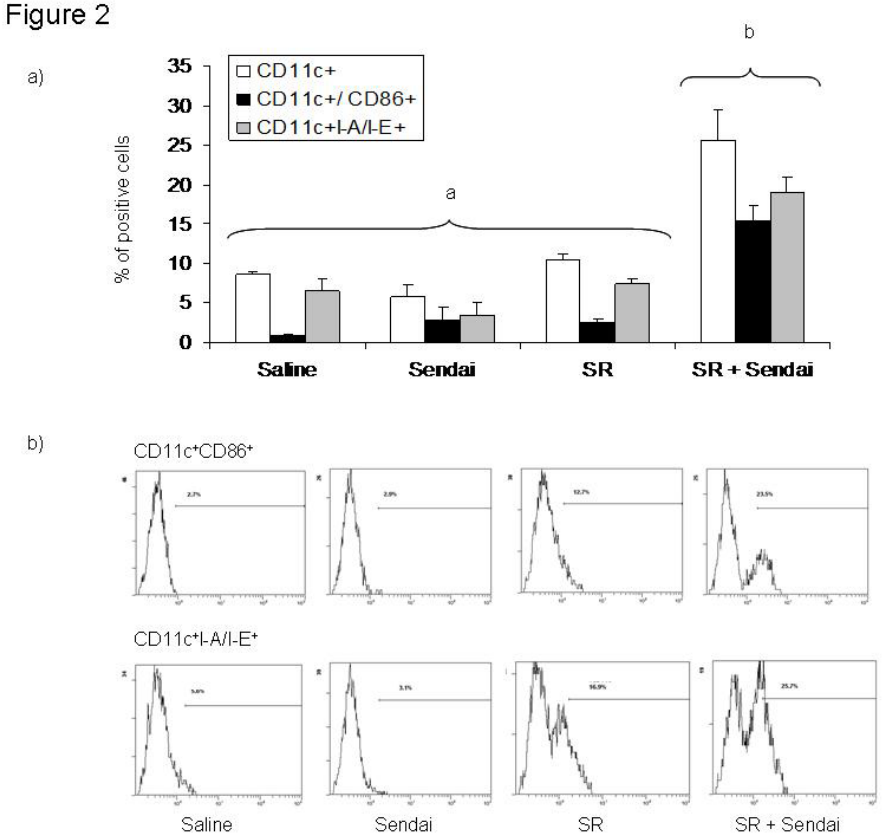


Figure 3: Immunofluorescence studies of lung section from each group of mice. Tissue sections were marked with anti-CD11c FITC (green) and double stained with anti-CD86 **(a-d)** or anti-I-A/I-E **(e-h)** alexa fluor (blue). Slides were blindly evaluated by three persons and intensity and quantity of marked cells were scored **(i)** (graded on an arbitrary scale, from 0 to 3: 0, no fluorescence; 1, low; 2, medium; 3, high. Intraclass correlations coefficients showed that evaluators agreed

for each markers (CD11c: 0.8913; CD86: 0.9469; I/A-I/E:0.9519). ($p < 0.02$, n = 8 mice per group).

Figure 3

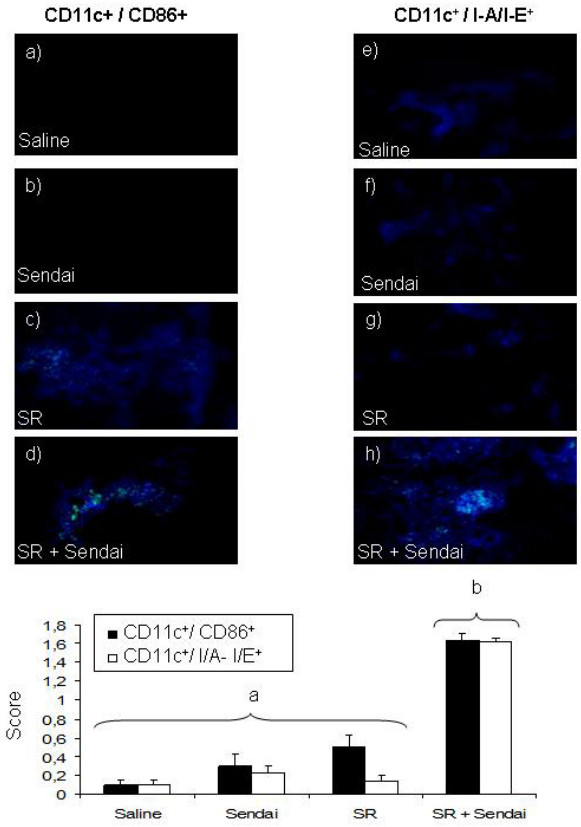
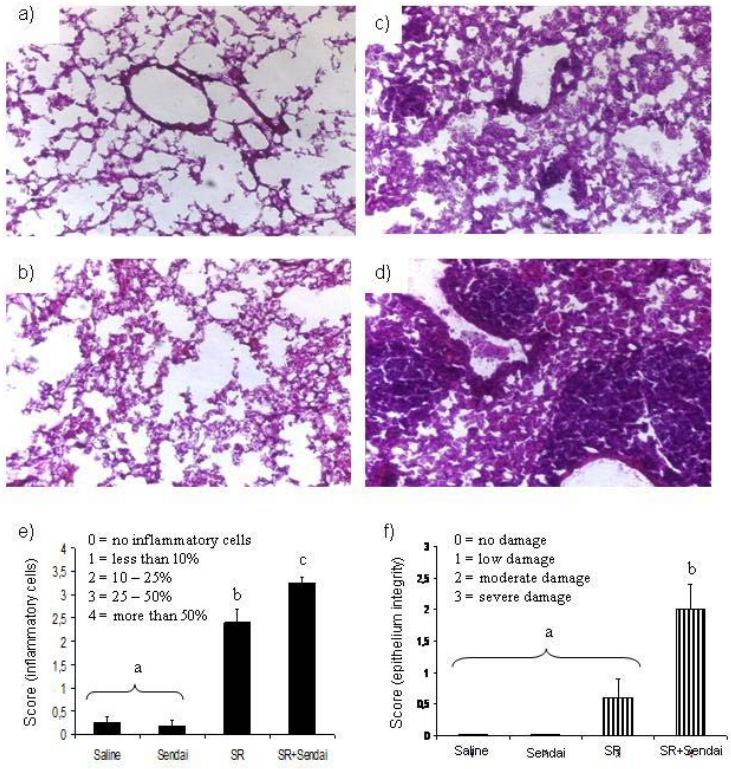


Figure 4: Histopathology results (X10) of Saline **(a)**, Sendai **(b)**, SR **(c)**, and SR + Sendai **(d)** instilled mice. SR+Sendai treatment induced a severe peribronchiolar and perivascular hyperplasia as well as important lung damage compared with SR-treated mice whereas Saline and Sendai treated-mice had normal lung. Results are expressed in **e)** as the total histologic score (graded on an arbitrary scale, from 0 to 4: 0, no inflammatory cells; 1, less than 10%; 2, 10-25%; 3, 25-50%; and 4, more than 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 ; and SR, 2.4 ± 0.3) ($p < 0.02$) **f)** Epithelial integrity was also scored from 0 to 3:

0, no damage and 3, severe epithelial damage. SR + Sendai mice show high epithelial damage compared to other groups. ($p < 0.01$, $n = 8$ mice per group).

Figure 4



Table

Table 1: Study design

<table>
<tr><td></td><td colspan="4">Groups</td></tr>
<tr><td>Week</td><td>Saline</td><td>SR</td><td>Sendai</td><td>SR + Sendai</td></tr>
<tr><td>1</td><td rowspan="12">50 µl of saline
3 days/week</td><td rowspan="12">50 µl of SR
(4mg/ml)
3 days/week</td><td>50 µl of saline
3 days/week</td><td>50 µl of SR
(4mg/ml)
3 days/week</td></tr>
<tr><td>2</td></tr>
<tr><td>3</td><td>50 µl of
Sendai virus
(50 HAU)</td><td>50 µl of
Sendai virus
(50 HAU)</td></tr>
<tr><td>4</td><td rowspan="9">50 µl of saline
3 days/week</td><td rowspan="9">50 µl of SR
(4mg/ml)
3 days/week</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td></tr>
<tr><td>7</td></tr>
<tr><td>8</td></tr>
<tr><td>9</td></tr>
<tr><td>10</td></tr>
<tr><td>11</td></tr>
<tr><td>12</td></tr>
<tr><td>13</td><td colspan="4">Sacrifice</td></tr>
</table>

Table legend

Table 1: Study design Four groups of mice were studied: Group 1 received intranasal instillations of saline; Group 2, instillations of SR during 12 weeks; Group 3, instillations of saline and a single infection with Sendai virus on week three; and Group 4, instillations of SR during 12 weeks with a single administration of Sendai virus on week three. Mice were sacrificed on week twelve.