min, but no preferential difference was observed between human Th1 and Th2 cells (Fig. 4).

In a mouse system, histone modifications associated with the silencing of the IL-4 and IL-13 genes in Th1 cells were reported (Koyanagi et al., 2005). We thus need to await further analyses addressing the problem of whether similar modifications may also exist in human Th1 cells. There were some differences among individuals in the methylation status of histone H3-K4 at the Th2 cytokine gene loci in 20 HV (Fig. 3 and Supplemental Figure 4). It would therefore be interesting to examine whether the levels of the histone H3-K4 methylation status are associated with the relative risk for certain Th2 immune diseases, including allergic asthma.

In summary, we herein analyzed the chromatin remodeling of the Th2 cytokine gene loci in human developing Th2 cells, particularly in regard to the methylation status of H3-K4, and thus found chromatin remodeling to occur throughout the Th2 cytokine gene loci including the intergenic regions accompanied with an increased GATA3 expression.

Acknowledgements

We thank Dr. Yoichi Suzuki for valuable suggestions and Ms. Hikari Asou, Satoko Norikane and Kaoru Sugaya and Mr. Toshihiro Ito for their excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid for: Scientific Research in Priority Areas #17016010 and #17047007; Scientific Research B #17390139, Scientific Research C #18590466; Grant-in-Aid for Young Scientists #17790318; Special Coordination Funds for Promoting Science and Technology), the Ministry of Health, Labor and Welfare (Japan), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (Japan), The Japan Health Science Foundation, Kanae Foundation and Uehara Memorial Foundation and Mochida Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2006.11.004.

References

Ansel, K.M., Djuretic, I., Tanasa, B., Rao, A., 2006. Regulation of Th2 differentiation and IL4 locus accessibility. Annu. Rev. Immunol. 24, 607–656.

Avni, O., Lee, D., Macian, F., Szabo, S.J., Glimcher, L.H., Rao, A., 2002. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. Nat. Immunol. 3, 643–651.

Barnes, P.J., Karin, M., 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. 336, 1066–1071.

Constant, S.L., Bottomly, K., 1997. Induction of Th1 and Th2 CD4+T cell responses: the alternative approaches. Annu. Rev. Immunol. 15, 297–322.

Das, J., Chen, C.H., Yang, L., Cohn, L., Ray, P., Ray, A., 2001. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat. Immunol. 2, 45–50.

Dong, C., Yang, D.D., Wysk, M., Whitmarsh, A.J., Davis, R.J., Flavell, R.A., 1998. Defective T cell differentiation in the absence of JNK1. Science 282, 2092–2095.

Gately, M.K., Renzetti, L.M., Magram, J., Stern, A.S., Adorini, L., Gubler, U., Presky, D.H., 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. 16, 495–521.

Inami, M., Yamashita, M., Tenda, Y., Hasegawa, A., Kimura, M., Hashimoto, K., Seki, N., Taniguchi, M., Nakayama, T., 2004. CD28 costimulation controls histone hyperacetylation of the interleukin 5 gene locus in developing th2 cells. J. Biol. Chem. 279, 23123–23133.

Ishikawa, E., Motohashi, S., Ishikawa, A., Ito, T., Uchida, T., Kaneko, T., Tanaka, Y., Horiguchi, S., Okamoto, Y., Fujisawa, T., Tsuboi, K., Taniguchi, M., Matsumura, A., Nakayama, T., 2005. Dendritic cell maturation by CD11c-T cells and Valpha24+ natural killer T-cell activation by alpha-galactosylceramide. Int. J. Cancer 117, 265–273.

Kimura, M.Y., Hosokawa, H., Yamashita, M., Hasegawa, A., Iwamura, C., Watarai, H., Taniguchi, M., Takagi, T., Ishii, S., Nakayama, T., 2005. Regulation of T helper type 2 cell differentiation by murine Schnurri-2. J. Exp. Med. 201, 397–408.

Koyanagi, M., Baguet, A., Martens, J., Margueron, R., Jenuwein, T., Bix, M., 2005. EZH2 and histone 3 trimethyl lysine 27 associated with IL4 and IL13 gene silencing in Th1 cells. J. Biol. Chem. 280, 31470–31477.

Lee, G.R., Kim, S.T., Spilianakis, C.G., Fields, P.E., Flavell, R.A., 2006. T helper cell differentiation: regulation by cis elements and epigenetics. Immunity 24, 369–379.

Lee, H.J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., Arai, N., 2000. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J. Exp. Med. 192, 105–115.

Lohning, M., Richter, A., Radbruch, A., 2002. Cytokine memory of T helper lymphocytes. Adv. Immunol. 80, 115–181.

Messi, M., Giacchetto, I., Nagata, K., Lanzavecchia, A., Natoli, G., Sallusto, F., 2003. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. Nat. Immunol. 4, 78–86.

Milne, T.A., Briggs, S.D., Brock, H.W., Martin, M.E., Gibbs, D., Allis, C.D., Hess, J.L., 2002. MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol. Cell 10, 1107–1117.

Mohrs, M., Blankespoor, C.M., Wang, Z.E., Loots, G.G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E.M., Locksley, R.M., 2001. Deletion of a coordinate regulator of type 2 cytokine expression in mice. Nat. Immunol. 2, 842–847.

Morinobu, A., Kanno, Y., O'Shea, J.J., 2004. Discrete roles for histone acetylation in human T helper 1 cell-specific gene expression. J. Biol. Chem. 279, 40640–40646.

Mosmann, T.R., Coffman, R.L., 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv. Immunol. 46, 111–147.

Mullen, A.C., Hutchins, A.S., High, F.A., Lee, H.W., Sykes, K.J., Chodosh, L.A., Reiner, S.L., 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. Nat. Immunol. 3, 652–658.

Murphy, K.M., Ouyang, W., Farrar, J.D., Yang, J., Ranganath, S., Asnagli, H., Afkarian, M., Murphy, T.L., 2000. Signaling and transcription in T helper development. Annu. Rev. Immunol. 18, 451–494.

Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C.M., Canaani, E., 2002. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol. Cell 10, 1119–1128.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., Paul, W.E., 1999. The IL-4 receptor: signaling mechanisms and biologic functions. Annu. Rev. Immunol. 17, 701–738.

O'Garra, A., 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 8, 275–283.

Omori, M., Yamashita, M., Inami, M., Ukai-Tadenuma, M., Kimura, M., Nigo, Y., Hosokawa, H., Hasegawa, A., Taniguchi, M., Nakayama, T., 2003. CD8 T cell-specific downregulation of histone hyperacetylation and gene activation of the IL-4 gene locus by ROG, repressor of GATA. Immunity 19, 281–294.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., Murphy, K.M., 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 9, 745–755.

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.A., Shen, H., Cereb, N., Yang, S.Y., Lindsten, T., Rossant, J., Hunter, C.A., Reiner,

S.L., 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science 302, 1041–1043.
Reiner, S.L., Locksley, R.M., 1995. The regulation of immunity to *Leishmania major*. Annu. Rev. Immunol. 13, 151–177.
Rincon, M., Enslen, H., Raingeaud, J., Recht, M., Zapton, T., Su, M.S., Penix, L.A., Davis, R.J., Flavell, R.A., 1998. Interferon-gamma expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. EMBO J. 17, 2817–2829.
Santangelo, S., Cousins, D.J., Winkelmann, N.E., Staynov, D.Z., 2002. DNA methylation changes at human Th2 cytokine genes coincide with DNase I hypersensitive site formation during CD4(+) T cell differentiation. J. Immunol. 169, 1893–1903.
Seder, R.A., Paul, W.E., 1994. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12, 635–673.
Shibata, Y., Kamata, T., Kimura, M., Yamashita, M., Wang, C.R., Murata, K., Miyazaki, M., Taniguchi, M., Watanabe, N., Nakayama, T., 2002. Ras activation in T cells determines the development of antigen-induced airway hyperresponsiveness and eosinophilic inflammation. J. Immunol. 169, 2134–2140.
Shirai, T., Magara, K.K., Motohashi, S., Yamashita, M., Kimura, M., Suwazomo, Y., Nogawa, K., Kuriyama, T., Taniguchi, M., Nakayama, T., 2003. TH1-biased immunity induced by exposure to Antarctic winter. J. Allergy Clin. Immunol. 111, 1353–1360.
Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100, 655–669.
Turner, B.M., 2002. Cellular memory and the histone code. Cell 111, 285–291.
Yamashita, M., Hirahara, K., Shinnakasu, R., Hosokawa, H., Norikane, S., Kimura, M.Y., Hasegawa, A., Nakayama, T., 2006. Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. Immunity 24, 611–622.
Yamashita, M., Kimura, M., Kubo, M., Shimizu, C., Tada, T., Perlmutter, R.M., Nakayama, T., 1999. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc. Natl. Acad. Sci. USA 96, 1024–1029.
Yamashita, M., Shinnakasu, R., Asou, H., Kimura, M., Hasegawa, A., Hashimoto, K., Hatano, N., Ogata, M., Nakayama, T., 2005. Ras-ERK MAPK cascade regulates GATA3 stability and Th2 differentiation through ubiquitin-proteasome pathway. J. Biol. Chem. 280, 29409–29419.
Yamashita, M., Ukai-Tadenuma, M., Kimura, M., Omori, M., Inami, M., Taniguchi, M., Nakayama, T., 2002. Identification of a conserved GATA3 response element upstream proximal from the interleukin-13 gene locus. J. Biol. Chem. 277, 42399–42408.
Yang, D.D., Conze, D., Whitmarsh, A.J., Barrett, T., Davis, R.J., Rincon, M., Flavell, R.A., 1998. Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity 9, 575–585.
Yano, S., Ghosh, P., Kusaba, H., Buchholz, M., Longo, D.L., 2003. Effect of promoter methylation on the regulation of IFN-gamma gene during in vitro differentiation of human peripheral blood T cells into a Th2 population. J. Immunol. 171, 2510–2516.
Zhang, D.H., Cohn, L., Ray, P., Bottomly, K., Ray, A., 1997. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J. Biol. Chem. 272, 21597–21603.
Zheng, W., Flavell, R.A., 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89, 587–596.

INSTITUT PASTEUR

Microbes and Infection 9 (2007) 364–374

www.elsevier.com/locate/micinf

Original article

Role of interferon-γ in Vα14+ natural killer T cell-mediated host defense against *Streptococcus pneumoniae* infection in murine lungs

Masashi Nakamatsu [a], Natsuo Yamamoto [a,1], Masumitsu Hatta [b], Chikara Nakasone [a], Takeshi Kinjo [a], Kazuya Miyagi [a], Kaori Uezu [a], Kiwamu Nakamura [a,b], Toshinori Nakayama [c], Masaru Taniguchi [d], Yoichiro Iwakura [e], Mitsuo Kaku [b,f,g], Jiro Fujita [a], Kazuyoshi Kawakami [g,h,*]

[a] *Department of Medicine and Therapeutics, Control and Prevention of Infectious Diseases, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan*
[b] *Department of Infection Control and Laboratory Diagnostics, Internal Medicine, Tohoku University Graduate School of Medicine, Tohoku University, Sendai, Japan*
[c] *Department of Medical Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan*
[d] *RIKEN Research Center for Allergy and Immunology, Yokohama, Japan*
[e] *Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan*
[f] *CRESCENDO (Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation) Project, Sendai, Japan*
[g] *Infection Control Research Center, Tohoku University Hospital, Tohoku University, Sendai, Japan*
[h] *Microbiology and Immunology, Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Tohoku University, Sendai, Japan*

Received 9 May 2006; accepted 18 December 2006
Available online 9 January 2007

Abstract

Previously, we demonstrated that Vα14+ NKT cells and IFN-γ are important upstream components in neutrophil-mediated host defense against infection with *Streptococcus pneumoniae*. In the present study, we extended these findings by elucidating the role of IFN-γ in this Vα14+ NKT cell-promoted process. Administration of recombinant IFN-γ to Jα18KO mice prolonged the shortened survival, promoted the attenuated clearance of bacteria and improved the reduced accumulation of neutrophils and synthesis of MIP-2 and TNF-α in the lungs, in comparison to wild-type (WT) mice. In addition, intravenous transfer of liver mononuclear cells (LMNC) from WT mice into Jα18KO mice resulted in complete recovery of the depleted responses listed above, whereas such effects were not detected when LMNC were obtained from IFN-γKO or Jα18KO mice. Activation of Vα14+ NKT cells by α-galactosylceramide (α-GalCer) significantly enhanced the clearance of bacteria, accumulation of neutrophils and synthesis of MIP-2 and TNF-α in the infected lungs; this effect was significantly inhibited by a neutralizing anti-IFN-γ antibody. Finally, in a flow cytometric analysis, TNF-α synthesis was detected largely by $CD11b^{bright+}$ cells in the infected lungs. Our results demonstrated that IFN-γ plays an important role in the neutrophil-mediated host protective responses against pneumococcal infection promoted by Vα14+ NKT cells.
© 2007 Elsevier Masson SAS. All rights reserved.

Keywords: NKT cells; Pneumococcal infection; Neutrophils; IFN-γ; Chemokine; Lung; Mouse

* Corresponding author. Microbiology and Immunology, Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Tohoku University, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan. Tel.: +81 (22) 717 7946; fax: 81 22 717 7910.
E-mail address: kawakami@mail.tains.tohoku.ac.jp (K. Kawakami).
[1] Present address: Allergy and Immunology Section, Department of Internal Medicine, Yale University School of Medicine New Haven, CT, USA.

1286-4579/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2006.12.003

1. Introduction

Streptococcus pneumoniae is an extracellular Gram-positive bacterium most frequently associated with community-acquired pneumonia, leading to severe pneumonia, bacteremia and meningitis in infants, elderly people and immunocompromised patients [1,2]. Pneumonia caused by this bacterium is characterized by massive infiltration of neutrophils into the alveolar spaces, which are central to the host defense against this infection via an oxygen radical-mediated killing mechanism [3]. The macrophage inflammatory protein (MIP)-2 is a C-X-C chemokine homologous to human interleukin (IL)-8, and plays a major role in attracting neutrophils to sites of inflammation in mice [4,5]. Tumor necrosis factor (TNF)-α is also involved in the accumulation of neutrophils by facilitating their adhesion to vascular endothelial cells through enhancing the expression of certain adhesion molecules [6,7]. These cytokines also act to promote the killing activity of neutrophils against infectious microorganisms [8,9]. In fact, more severe pneumonia occurs when MIP-2 [10,11] and TNF-α [12,13] are suppressed, due to impaired recruitment of inflammatory leukocytes to the infected tissues [4,5,10–13].

Interferon (IFN)-γ has been extensively studied as a mediator of host defenses against intracellular microorganisms [14–17]. Mice with a targeted disruption of the gene for this cytokine are highly prone to infection with such bacteria. In addition, IFN-γ is involved in the host protective responses against extracellular microorganisms [18,19]. Recently, we defined IFN-γ as a critical cytokine in the development of neutrophil-mediated host defense to *S. pneumoniae* infection [20]. IFN-γ promotes the synthesis of MIP-2 and TNF-α and consequently the accumulation of neutrophils in lungs infected with *S. pneumoniae*.

Natural killer T (NKT) cells express both NK cell markers and T cell antigen receptors composed of an invariant α chain with Vα14–Jα18 and a highly skewed β chain with Vβ8.2, Vβ7 and Vβ2. These cells play a regulatory role in the immunological response under various pathological conditions, including tumor formation, autoimmune diseases, allergy and infection, by rapidly secreting large amounts of IFN-γ and IL-4 [21–23]. Previous studies reported the involvement of this particular lymphocyte subset in the host defense against infectious pathogens [24–30]. In our recent study [25], deficiency of Vα14+ NKT cells resulted in increased susceptibility to infection with *S. pneumoniae*, which was associated with reduced synthesis of MIP-2 and TNF-α and an attenuated recruitment of neutrophils into the infected tissues. However, it remains to be understood how the Vα14+ NKT cells function in these responses. The present study was designed to elucidate the role of IFN-γ in this mechanism and present evidence that this cytokine may act downstream of Vα14+ NKT cells in the neutrophil response and host defense against *S. pneumoniae* infection.

2. Materials and methods

2.1. Animals

Vα14+ NKT cell-deficient (Jα18KO) mice were established by targeted deletion of the Jα18 gene segment [31]. IFN-γ gene-disrupted (GKO) mice were established as described previously [32]. These mice were back-crossed more than eight times with C57Bl/6 mice, purchased from Charles River Japan (Osaka, Japan) and used as control WT animals for the KO mice. In some experiments, C57Bl/6 mice were obtained from the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine. Mice were bred in a pathogen-free environment in the Laboratory Animal Center for Biomedical Science, University of the Ryukyus. All mice were used for experiments at 6–15 weeks of age. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of University of the Ryukyus and Tohoku University.

2.2. Bacteria

A serotype 3, clinical strain of *S. pneumoniae*, designated as URF918, was established from a patient with pneumococcal pneumonia. The bacteria were cultured in Todd–Hewitt broth (Difco, Detroit, MI) at 37 °C in a 5% CO_2 incubator, harvested at 6 h, at mid-log phase of growth, and then washed twice in phosphate-buffered saline (PBS). The inoculum was prepared at $2–6 \times 10^7$ CFU/ml. To induce pulmonary infection, mice were anesthetized by intraperitoneal injection of 70 mg/kg of pentobarbital (Abbott Lab., North Chicago, IL) and restrained on a small board. Live *S. pneumoniae* were inoculated at 50 μl per mouse by insertion of a 24-gauge blunt needle into and parallel to the trachea. In every experiment, 10 times dilution of quantification culture was performed to confirm the inoculation dose.

2.3. Enumeration of viable S. pneumoniae

Mice were sacrificed on day 3 post-infection. Their lungs were carefully dissected and excised, then separately homogenized in 10 ml of half saline by teasing with a stainless-steel mesh at room temperature. The homogenates, appropriately diluted with half saline, were inoculated at 100 μl on 3% sheep blood Muller–Hinton agar plates and cultured for 18 h. The number of subsequent bacterial colonies was recorded.

2.4. Analysis of neutrophils in BAL fluids

Mice were sacrificed 6 h after infection and samples of bronchoalveolar lavage (BAL) fluids were collected as described below. Briefly, after bleeding under anesthesia with ether, the chest was opened and the trachea was cannulated with the outer sheath of a 22-G I.V. catheter/needle unit (Becton Dickinson Vascular Access, Sandy, UT), followed by lavage of the lung three times with 1 ml of chilled PBS. Approximately 1×10^5 cells were centrifuged onto a glass slide at 800 rpm for 3 min using an Auto Smear CF-12D (Sakura Co., Tokyo), and stained using May–Giemsa technique. The total number of neutrophils was estimated by multiplying the total leukocyte number by the proportion of neutrophils in 500 cells.

2.5. Preparation of liver mononuclear cells

Liver mononuclear cells (LMNC) were prepared as described previously with some modification [33]. Briefly, livers were excised from mice anesthetized with ether and teased apart using a stainless-steel mesh in RPMI1640 medium (Nipro, Osaka, Japan) supplemented with 5 mM HEPES and 2% fetal calf serum (FCS) (Cansera, Rexdale, Ontario, Canada). After washing, the pellets were suspended in 15 ml of 35% Percoll solution (Pharmacia, Uppsala, Sweden) containing heparin (100 U/ml) and centrifuged at 2000 rpm for 15 min at 20 °C. The pellet was then resuspended in red blood cell lysis solution (155 mM NH_4Cl and 17 mM Tris, pH 7.2) and washed twice with RPMI1640 containing PBS. The LMNC were injected intravenously at 1×10^6/mouse 1 day before infection.

2.6. Treatment with α-GalCer

α-Galactocylceramide (α-GalCer) was kindly provided by Kirin Brewery Co. (Gunma, Japan) and prepared as described previously [34,35]. The stock solution of α-GalCer (200 μg/mL in 0.5% polysorbate 20 in normal saline) was diluted to 10 μg/mL with normal saline. Polysorbate 20 solution (0.03% in normal saline) was used as a control vehicle solution. α-GalCer (1 ng per mouse) or equivalent volume of control solution was injected intratracheally on the day of infection.

2.7. Administration of recombinant IFN-γ and neutralizing anti-IFN-γ mAb

Mice were injected intraperitoneally with mouse recombinant (r)IFN-γ [PeproTech, Inc. (Rocky Hill, NJ)] at 5000 U/mouse at 12 and 1 h before and at 1, 3, 6, 24, 36 and 48 h after infection. To block endogenously synthesized IFN-γ, mice were injected intratracheally with mAb against this cytokine (clone 37895; R&D Systems, Minneapolis, MN) at 25 μg/mouse at the same time as the infection. Rat IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was used as a control antibody.

2.8. Measurement of cytokine concentrations

The concentrations of MIP-2 and TNF-α in the lung homogenates were measured by the respective ELISA kits (R&D Systems). The detection limits of assays for MIP-2 and TNF-α were 1.5 and 5.1 pg/ml, respectively.

2.9. Preparation of pulmonary intraparenchymal leukocytes

Pulmonary intraparenchymal leukocytes were prepared as described previously [36]. Briefly, the chest of the mouse was opened, and the lung vascular bed was flushed by injecting 3 ml of chilled physiological saline into the right ventricle. The lungs were then excised and washed in physiological saline. The lungs, teased with a stainless-steel mesh, were incubated in RPMI1640 medium with 10 mM HEPES and 5% FCS, containing 20 U/ml collagenase (Sigma Chemical Co., St. Louis, MO) and 1 μg/mL DNaseI (Sigma). After incubation for 60 min at 37 °C with vigorous shaking, the tissue fragments and the majority of dead cells were removed by passing through the 40-μm nylon mesh. After centrifugation, the cell pellet was resuspended in 4 ml of 40% (v/v) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% (v/v) Percoll. After centrifugation at 600 × *g* for 20 min at 15 °C, the cells at the interface were collected, washed three times and counted with a hemocytometer. The obtained cells contained lymphocytes, macrophages and neutrophils.

2.10. Analysis of intracellular TNF-α expression

The lung leukocytes were stained with phycoerythrin (PE)-conjugated anti-CD11b, -NK1.1 and -TCRγδ mAb (clone M1/70, PK136 and GL3, respectively; BD Pharmingen, San Diego, CA) or control IgG. After washing twice, the cells were incubated in the presence of cytofix/cytoperm (BD Biosciences, San Jose, CA), washed twice in BD Perm/wash solution and stained with fluorescein isothiocyanate (FITC)-conjugated anti-TNF-α mAb (clone MP6-XT22; BD Pharmingen) or control IgG. The stained cells were analyzed using an FACS Caliber flow cytometer (BD Pharmingen).

2.11. Statistical analysis

Analysis of data was conducted using StatView II software (Abacus Concept, Inc., Berkeley, CA) on a Macintosh computer. Data are expressed as mean ± standard deviation (SD). Statistical analysis between groups was performed using ANOVA test with a post hoc analysis (Fisher PLSD test). Survival data were analyzed using the generalized Wilcoxon test. A *p*-value less than 0.05 was considered significant.

3. Results

3.1. IFN-γ restoration of the reduced host resistance to pneumococcal infection in Vα14+ NKT cell-deficient mice

To elucidate whether IFN-γ acts in the neutrophil-mediated host defense mechanism potentiated by Vα14+ NKT cells, we initially examined the effect of administrating this cytokine on the clinical course of infection caused by *S. pneumoniae.* As shown in Fig. 1A, 25% of WT mice died on day 8 while the others survived the observation period, whereas Jα18KO mice commenced to die on day 2 and all died within 9 days after infection. Interestingly, administration of rIFN-γ significantly extended the survival time of Jα18KO mice compared to WT mice. Furthermore, we tested the effect of rIFN-γ on clearing *S. pneumoniae* from the lungs of Jα18KO mice. As shown in Fig. 1B, the number of live bacteria was significantly higher in Jα18KO mice than in WT mice, and the administration of this cytokine significantly reduced bacterial counts in the Jα18KO mice, although this decrease was not pronounced.

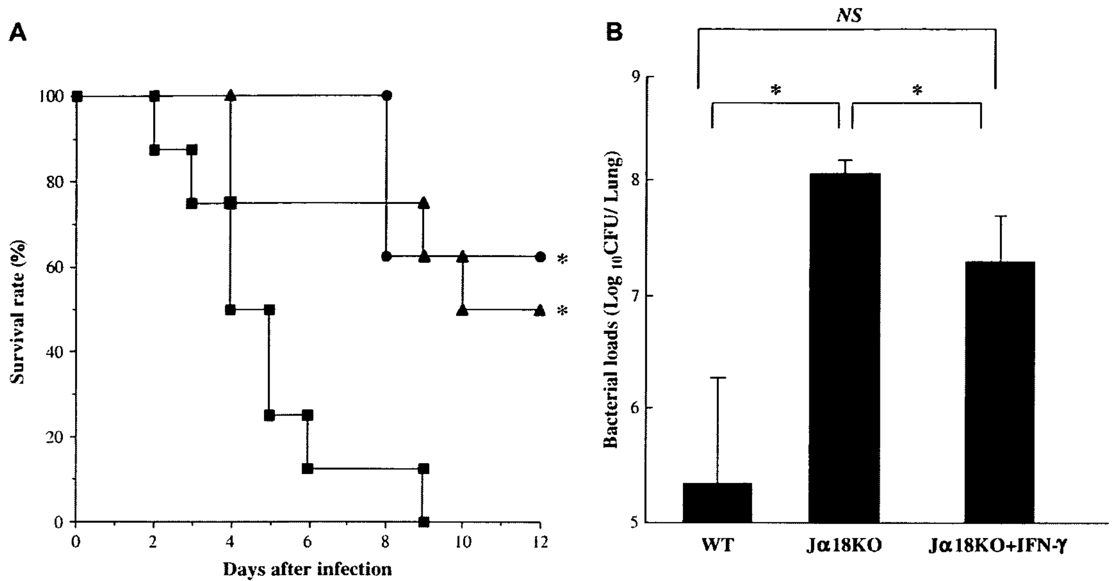

Fig. 1. Administration of rIFN-γ results in recovery of the impaired host resistance of *S. pneumoniae* in Jα18KO mice. WT and Jα18KO mice were infected intratracheally with *S. pneumoniae*. Jα18KO mice received intraperitoneal injections of PBS or rIFN-γ. (A) The number of live mice was noted daily. Each group consisted of eight mice. Circles, WT mice; squares, Jα18KO mice treated with PBS; and triangles, Jα18KO mice with rIFN-γ. $*p < 0.05$, compared with Jα18KO mice treated with PBS. (B) The number of live colonies in the lung homogenates was counted on day 3 after infection. Each bar represents the mean ± SD of six mice. Similar results were obtained in three experiments. NS, not significant; $*p < 0.05$.

3.2. Effect of IFN-γ administration on the reduced neutrophil accumulation and cytokine synthesis in Vα14+ NKT cell-deficient mice

We reported previously that Vα14+ NKT cell defect was associated with reduced production of MIP-2 and TNF-α and attenuated recruitment of neutrophils into the lungs infected with *S. pneumoniae* [25]. These findings suggest that Vα14+ NKT cells may contribute to the neutrophil-mediated host responses by affecting the production of IFN-γ. Therefore, we next examined whether the administration of rIFN-γ influenced these responses that were attenuated in Jα18KO mice. The

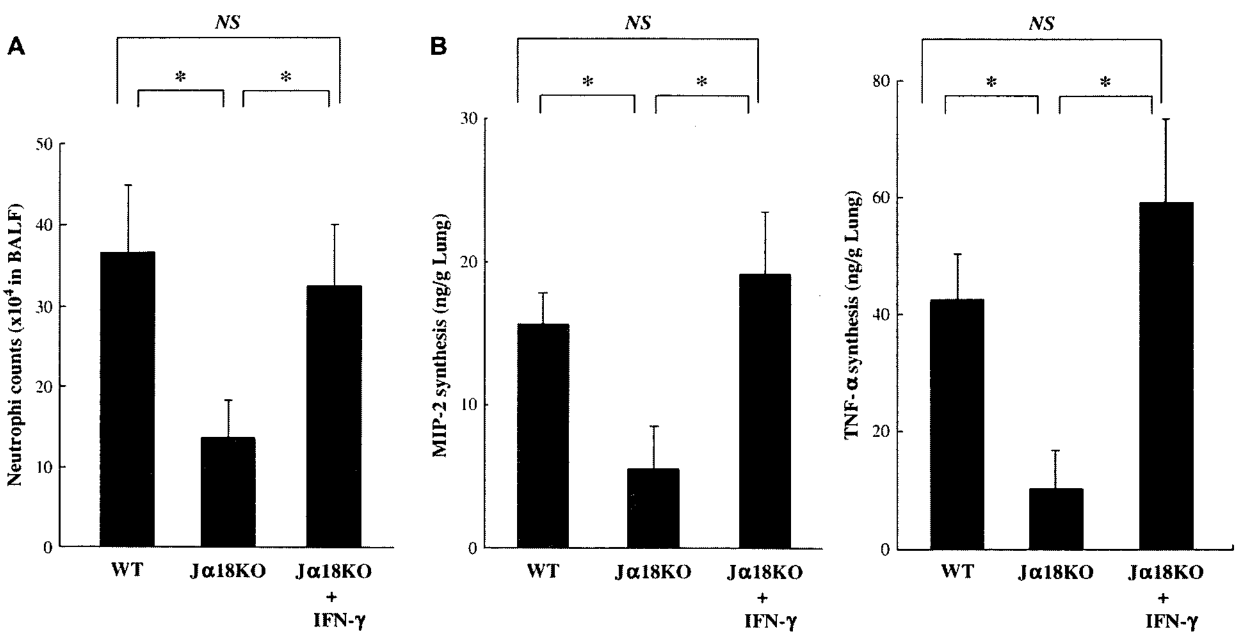

Fig. 2. Administration of IFN-γ recovers the reduced neutrophil responses to *S. pneumoniae* in Jα18KO. WT and Jα18KO mice were infected intratracheally with *S. pneumoniae*. Jα18KO mice received intraperitoneal injections of PBS or rIFN-γ. The number of neutrophils in the BAL fluids (A) and the concentrations of MIP-2 and TNF-α in the lung homogenates (B) were measured at 6 and 3 h after infection, respectively. Each bar represents the mean ± SD of six mice. Similar results were obtained in three experiments. NS, not significant; $*p < 0.05$.

number of neutrophils in BAL fluids was significantly lower in Jα18KO mice than in WT mice at 6 h after infection with *S. pneumoniae*, and the administration of rIFN-γ recovered the reduced accumulation of neutrophils in Jα18KO mice compared to WT mice (Fig. 2A). Similarly, synthesis of MIP-2 and TNF-α in the lungs at 3 h after infection was significantly lower in Jα18KO mice than in WT mice, and the same treatment with rIFN-γ completely rescued these attenuated responses (Fig. 2B). Considered collectively, these results suggest that IFN-γ may act downstream of Vα14+ NKT cells in the neutrophil-mediated host defense to this infection.

3.3. IFN-γ is an essential cytokine in Vα14+ NKT cell-mediated host defenses against pneumococcal infection

To define the role of Vα14+ NKT cells in immunity against infection, we transferred LMNC into Jα18KO mice and tested for clearance of *S. pneumoniae*, accumulation of neutrophils and synthesis of MIP-2 and TNF-α in the lungs. As shown in Fig. 3A, the number of live bacteria was significantly higher in Jα18KO mice than in WT mice on day 3 after infection. Transfer of LMNC from WT mice significantly promoted the clearance of bacteria in Jα18KO, whereas such an effect

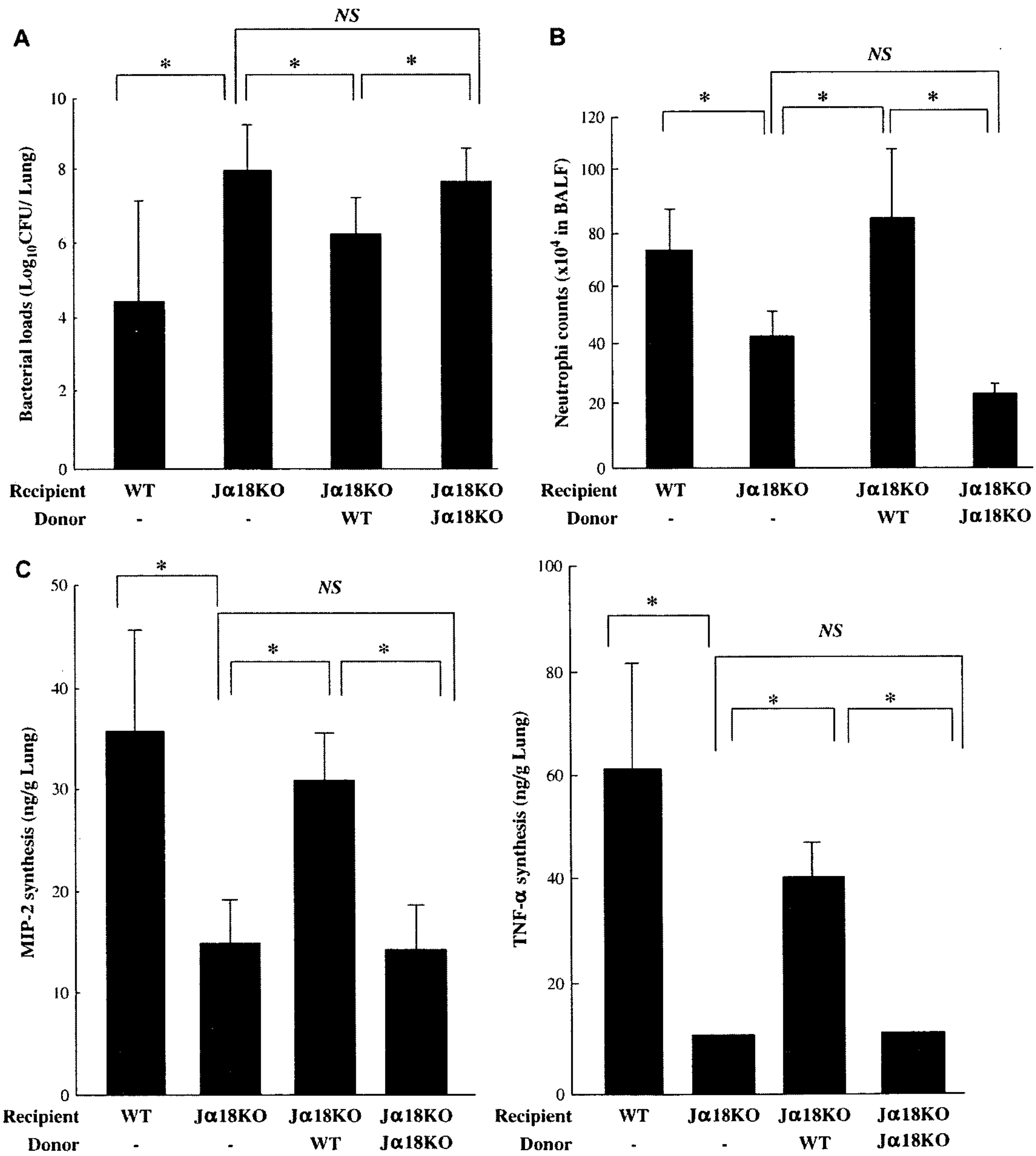

Fig. 3. Transfer of LMNC from Jα18KO mice does not recover the impaired host protective responses in Jα18KO mice. WT and Jα18KO mice were infected intratracheally with *S. pneumoniae*. Jα18KO mice received intraperitoneal injections of LMNC (1×10^6/mouse) derived from WT or Jα18KO mice 12 h before infection. The live colonies in the lung homogenates were estimated: (A). neutrophils in the BAL fluids (B), and concentration of MIP-2 and TNF-α in the lung homogenates (C) were measured on day 3, at 6 and 3 h after infection, respectively. Each bar represents the mean ± SD of six mice. NS, not significant; $*p < 0.05$.

was not observed when the LMNC were sourced from Jα18KO mice. Similarly, transfer of LMNC from WT mice almost completely rescued the impaired accumulation of neutrophils and reduced synthesis of MIP-2 and TNF-α in the lungs of Jα18KO mice. In contrast, such a recovery effect was completely abolished when the transferred LMNC were prepared from Jα18KO mice (Fig. 3B and C).

In other experiments, LMNC obtained from WT and GKO mice were transferred to Jα18KO mice to define the contribution of IFN-γ to the Vα14+ NKT cell-mediated host protective responses. Clearance of *S. pneumoniae* from the affected lungs was significantly promoted by transfer of LMNC from WT mice, whereas no such effect was observed with GKO mice-derived cells (Fig. 4A). In addition, transfer of WT-LMNC recovered the decreased accumulation of neutrophils and attenuated synthesis of MIP-2 and TNF-α in Jα18KO mice to a level comparable with that in WT mice. In contrast, such a recovery effect was not detected when the transfer was conducted using LMNC obtained from GKO mice (Fig. 4B and C). These results clearly indicate that Vα14+ NKT cells contribute to the neutrophil-mediated host protective responses against pneumococcal infection of lungs in an IFN-γ-dependent manner.

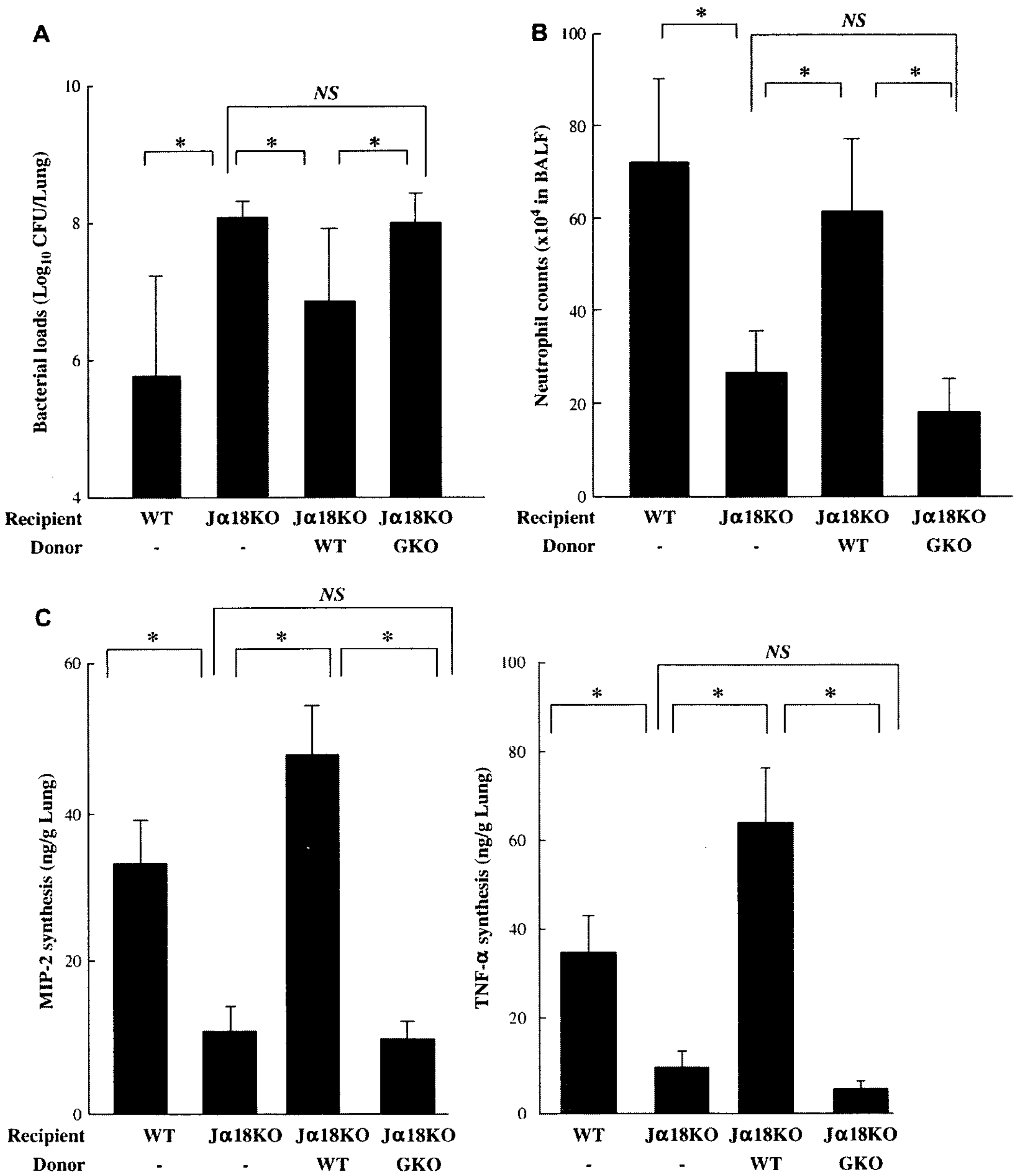

Fig. 4. Transfer of LMNC from GKO mice does not recover the impaired host protective responses in Jα18KO mice. WT and Jα18KO mice were infected intratracheally with *S. pneumoniae*. Jα18KO mice received intraperitoneal injections of LMNC (1×10^6/mouse) derived from WT or GKO mice 12 h before infection. The live colonies in the lung homogenates were estimated: (A) neutrophils in the BAL fluids (B) and concentration of MIP-2 and TNF-α in the lung homogenates (C) were measured on day 3, at 6 and 3 h after infection, respectively. Each bar represents the mean ± SD of six mice. Similar results were obtained in three experiments. NS, not significant; $*p < 0.05$.

3.4. Involvement of IFN-γ in host protection against pneumococcal infection caused by activation of Vα14+ NKT cells

In our earlier study [25], activation of Vα14+ NKT cells using α-GalCer promoted the clearance of *S. pneumoniae* from the lungs. Here, we showed that α-GalCer significantly increased the number of neutrophils and production of MIP-2 and TNF-α in the lungs at 6 and 3 h after infection, respectively, in contrast to uninfected mice in which such effect was either absent or only marginally observed in uninfected mice (Fig. 5A and B). Taken together, these observations suggest that IFN-γ may be involved in the neutrophil-mediated inflammatory responses caused by α-GalCer activation of Vα14+ NKT cells. To address this possibility, we examined the effect of neutralizing mAb against this cytokine on the clearance of bacteria, accumulation of neutrophils and synthesis of MIP-2 and TNF-α promoted by Vα14+ NKT cell activation at 6 and 3 h after infection, respectively. As shown in Fig. 6A–C, the augmenting effects of α-GalCer treatment on these parameters were significantly inhibited by treatment with anti-IFN-γ mAb, when compared to that of control rat IgG.

3.5. Production of TNF-α by CD11b$^{bright+}$ cells in lung after pneumococcal infection

Finally, we elucidated the cellular source of TNF-α production in the lung after infection with *S. pneumoniae* by detecting intracellular staining with Ab against this cytokine. As shown in Fig. 7, TNF-α was considerably detected in CD11b$^{bright+}$ cells, but not in CD11b^{dull+} and CD11b^{-} cells, in the lung leukocytes at 3 h post-infection, whereas such production was not found in any population before infection. In addition, this cytokine was not detected in NK1.1^{+} and TCRγδ^{+} cells in both infected and uninfected lungs (data not shown). These results indicated that CD11b$^{bright+}$ cells were a major source of TNF-α production at the early stage after pneumococcal infection.

4. Discussion

The current study demonstrated that (1) administration of rIFN-γ improved the shortened mice-survival time, impaired clearance of bacteria, reduced accumulation of neutrophils and attenuated production of MIP-2 and TNF-α in the lungs

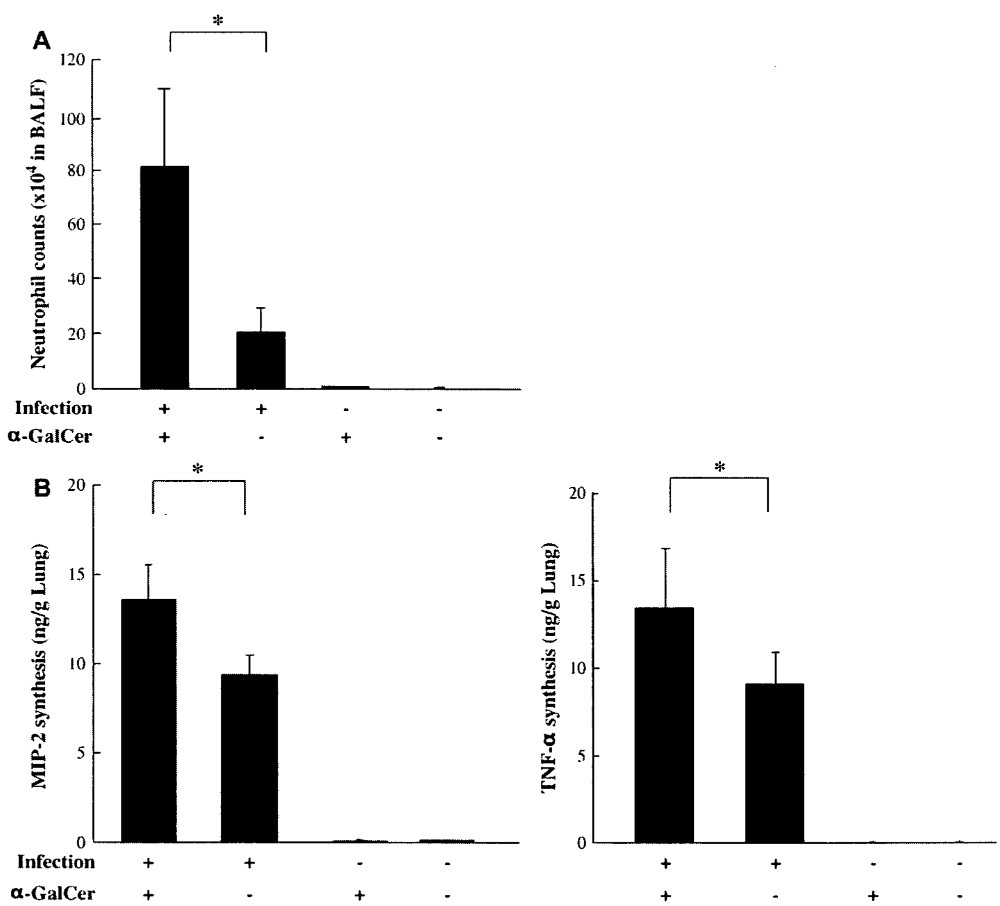

Fig. 5. α-GalCer induces neutrophil responses in the lungs after infection with *S. pneumoniae*. WT mice were administered intratracheally with *S. pneumoniae* or normal saline. These infected and uninfected mice received an injection of α-GalCer (1 ng/mouse) or equivalent volume of vehicle. The number of neutrophils in the BAL fluids (A) and concentration of MIP-2 and TNF-α in the lung homogenates (B) were measured at 6 and 3 h after infection, respectively. Each bar represents the mean ± SD of six mice. Similar results were obtained in three experiments. *$p < 0.05$.

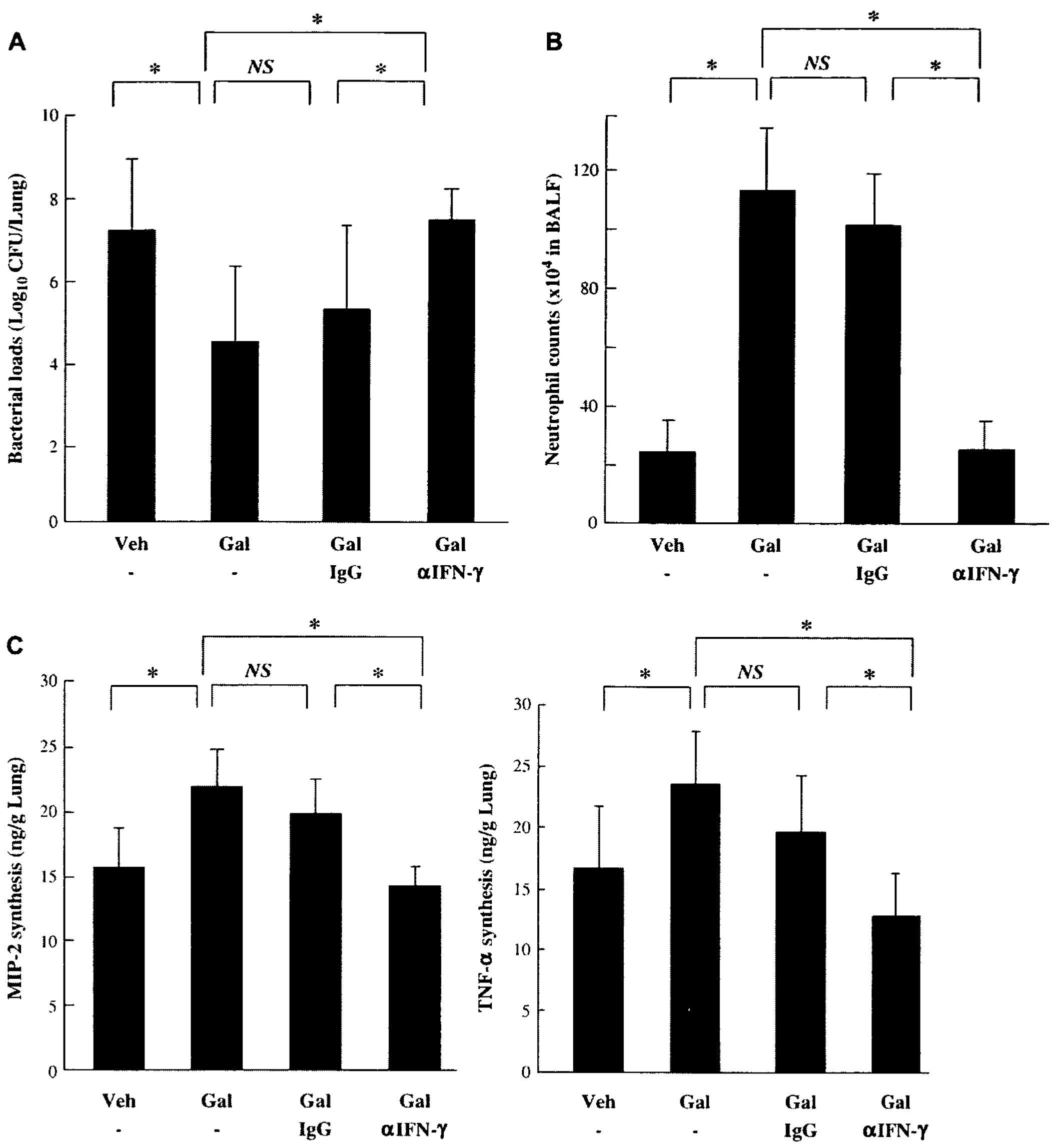

Fig. 6. IFN-γ contributes to α-GalCer induction of neutrophil responses after infection with *S. pneumoniae*. WT mice were infected intratracheally with *S. pneumoniae*. These mice received an injection of α-GalCer (1 ng/mouse) or equivalent volume of vehicle. α-GalCer-treated mice were administered with anti-IFN-γ mAb or rat IgG at the same time of infection. The number of live colonies (A), neutrophils in the BAL fluids (B) and concentration of MIP-2 and TNF-α in the lung homogenates (C) were measured at 6 and 3 h after infection, respectively. Each bar represents the mean ± SD of six mice. Similar results were obtained in two (A) and three experiments (B, C). Veh, vehicle; Gal, α-GalCer; and αIFN-γ, anti-IFN-γ mAb. NS, not significant; *$p < 0.05$.

of Jα18KO mice; (2) transfer of LMNC from WT mice, but not from Jα18KO and GKO mice, rescued these responses in Jα18KO mice; (3) activation of Vα14+ NKT cells by α-GalCer promoted bacterial clearance and neutrophil responses in the infected WT mice, and these effects were significantly inhibited by treatment with neutralizing anti-IFN-γ mAb; and (4) TNF-α was produced largely by $CD11b^{bright+}$ cells in the lung leukocytes. These results suggest that IFN-γ may act downstream of Vα14+ NKT cells to promote the neutrophil-mediated inflammatory response and host defenses against infection with *S. pneumoniae*.

Previous reports identified IFN-γ as an important cytokine in various immune responses involving Vα14+ NKT cells [37–42], suggesting that this cytokine may contribute to the host protective responses promoted by Vα14+ NKT cells in our experimental system. In agreement with these results, we recently showed that IFN-γ plays an important role upstream of the neutrophil response in the host resistance mechanism [20], although conflicting data were reported from other laboratories [43]. Therefore, we tested the effect of rIFN-γ administration on the survival of infected mice, clearance of the infecting bacterium, accumulation of neutrophils, and synthesis of MIP-2 and TNF-α in Jα18KO mice. All these weakened responses in the mutant mice were almost completely recovered by the treatment, consistent with our hypothesis that IFN-γ functions as a downstream molecule following activation of Vα14+ NKT cells.

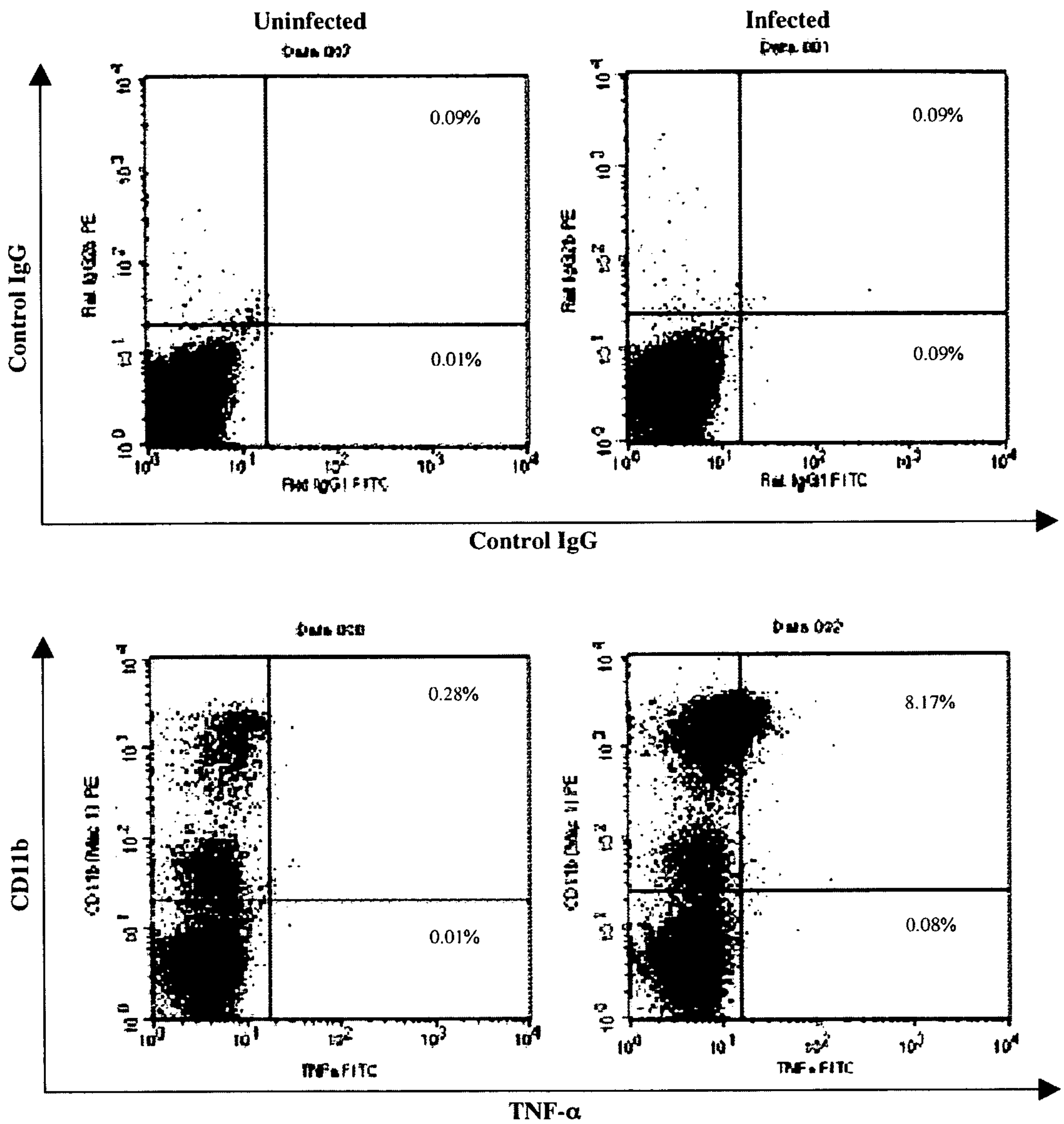

Fig. 7. TNF-α production by CD11b[bright+] cells in the lungs after pneumococcal infection. WT mice were infected intratracheally with *S. pneumoniae*. The lung leukocytes were prepared and stained with FITC-anti-TNF-α and PE-anti-CD11b mAbs or each isotype-matched IgG before and 3 h after infection. The leukocyte population was analyzed by flow cytometry. Similar results were obtained in two experiments.

It is true that we could not exclude that added rIFN-γ triggered alternative, Vα14+ NKT cells-dependent pathways for activation of the neutrophil-mediated host protective responses, rather than recovering the downstream events triggered by these cells. To address this caveat, we also further tested the effect of transferring LMNC, containing 20–30% of NKT cells, on the impaired neutrophil responses and host defense to infection in Jα18KO mice. The transfer of LMNC from WT mice almost completely recovered the synthesis of MIP-2 and TNF-α, accumulation of neutrophils and clearance of *S. pneumoniae* in the infected lungs, whereas no such effect was observed when LMNC were prepared from either Jα18KO or GKO mice. On the other hand, activation of Vα14+ NKT cells by α-GalCer administration accelerates the clearance of *S. pneumoniae* from the lungs, as recently shown by our group [25]. In addition, the current and our recent studies [44] demonstrated that the administration of α-GalCer promoted the early production of IFN-γ, MIP-2, and TNF-α, and the migration of neutrophils at the site of infection. In the current study, we also showed that such effects were significantly suppressed by treating the infected and α-GalCer-administered mice with neutralizing anti-IFN-γ mAb. Considered collectively, these observations provided additional evidence to support our hypothesis that Vα14+ NKT cells adopt IFN-γ to connect the initial events of *S. pneumoniae* infection to the consequent downstream cascade for developing the neutrophil-mediated host protective responses.

Given that IFN-γ-induced downstream events in the mice lungs were promoted by the Vα14+ NKT cells, we could have predicted that production of this cytokine was reduced in Jα18KO mice compared to that in WT mice. In most experiments, IFN-γ was not detectable in either BAL fluids or lung homogenates from both mouse strains at the earlier stages (1–3 h) of infection, as also shown in our recent study [44]. However, in some cases, a small amount of IFN-γ was detected in the lung homogenates, but at a lower level in the Jα18KO mice than in WT mice (data not shown). Alternatively, we examined the intracellular expression of IFN-γ in Vα14+ NKT cells, identified as a lymphocyte subset binding to α-GalCer-CD1d tetramer, at 1 or 2 h after infection. However, the proportion of Vα14+ NKT cells was too low, at 0.2–0.4% of the total number of lymphocytes, to accurately discriminate between IFN-γ-positive and -negative cells ([25] and our unpublished data). In addition, we measured the expression of IFN-γ mRNA in the lung homogenates using a real-time PCR method. We did not reproducibly detect such increase at 1 and 3 h post-infections, compared to that before infection, in both Jα18KO and WT mice (data not shown). Thus, we were unable to make a definite conclusion in the effect of Vα14+ NKT cell deficiency on IFN-γ production caused by *S. pneumoniae* infection.

In a flow cytometric analysis, TNF-α production was detected in $CD11b^{bright+}$ cells, but not in $CD11b^{dull+}$ and $CD11b^{-}$ cells, after infection with *S. pneumoniae*. By contrast, neither $NK1.1^{+}$ cells nor $TCR\gamma\delta^{+}$ cells expressed the intracellular production of this cytokine. In a previous report by Gonzalez-Juarrero and co-workers [45], $CD11b^{bright+}$ and $CD11b^{dull+}$ cells are identified to be dendritic cells or neutrophils and macrophages or NK cells, respectively, in the lungs after infection with *Mycobacterium tuberculosis*. Alveolar macrophages do not express this molecule. These previous findings suggested that TNF-α-producing cells were dendritic cells or neutrophils, rather than innate immune lymphocytes such as NK, NKT and γδ T cells, in our study, although a possible contribution of macrophages could not be excluded.

In conclusion, we revealed the possible contribution of IFN-γ as a downstream molecule in Vα14+ NKT cells that promote neutrophil-mediated host protective responses against *S. pneumoniae* infection, although the precise mechanism remains to be further elucidated. The present study has important implications for our understanding of the pathogenic mechanisms of this bacterial pathogen, and suggests the usefulness of α-GalCer, a specific activator of NKT cells, in clinical treatment and as an adjuvant vaccine against this infectious disease.

Acknowledgments

The authors thank Dr. Kronenberg, La Jolla Institute for Allergy and Immunology, for the kind gift of α-GalCer-loaded and -unloaded control CD1d tetramer. This work was supported in part by a Grant-in-aid for Science Research (C) (16590366) from the Ministry of Education, Science and Culture and Tohoku University 21st COE program "CRESCENDO".

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micinf.2006.12.003.

References

[1] V. Gant, S. Parton, Community-acquired pneumonia, Curr. Opin. Pulm. Med. 6 (2000) 226–233.
[2] T.J. Marrie, Pneumococcal pneumonia: epidemiology and clinical features, Semin. Respir. Infect. 14 (1999) 227–236.
[3] D. Musher, M. Cohen, C. Baker, Immune responses to extracellular bacteria, in: R.R. Rich, T.A. Fleisher, B.D. Schwartz, W.T. Shearer, W. Strober (Eds.), Clinical Immunology. Principles and Practice, Mosby-Year Book, Inc., St. Louis, MO, 1996, pp. 479–502.
[4] K.E. Driscoll, D.G. Hassenbein, B.W. Howard, R.J. Isfort, D. Cody, M.H. Tindal, M. Suchanek, J.M. Carter, Cloning, expression, and functional characterization of rat MIP-2: a neutrophil chemoattractant and epithelial cell mitogen, J. Leukoc. Biol. 58 (1995) 359–364.
[5] D. Rossi, A. Zlotonik, The biology of chemokines and their receptors, Annu. Rev. Immunol. 18 (2000) 217–242.
[6] F. Mackay, H. Loetscher, D. Stueber, G. Gehr, W. Lesslauer, Tumor necrosis factor-alpha (TNF-alpha)-induced cells adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55, J. Exp. Med. 177 (1993) 1277–1286.
[7] T. Collins, M.A. Read, A.S. Neish, M.Z. Whitley, D. Thanos, T. Maniatis, Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers, FASEB J. 9 (1995) 899–909.
[8] E. Broug-Holub, G.B. Toews, J.F. van Iwaarden, R.M. Strieter, S.L. Kunkel, R. Paine 3rd., T.J. Standiford, Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumonia*: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival, Infect. Immun. 65 (1997) 1139–1146.
[9] A. Ferrante, A.J. Martin, E.J. Bates, D.H. Goh, D.P. Harvey, D. Parsons, D.A. Rathjen, G. Russ, J.M. Dayer, Killing of *Staphylococcus aureus* by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation, J. Immunol. 151 (1993) 4821–4828.
[10] M.J. Greenberger, R.M. Strieter, S.L. Kunkel, J.M. Danforth, L.L. Laichalk, D.C. McGillicuddy, T.J. Standiford, Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella pneumonia*, J. Infect. Dis. 173 (1996) 159–165.
[11] W.C. Tsai, R.M. Strieter, B. Mehard, M.W. Newstead, X. Zeng, T.J. Standiford, CXC chemokine receptor CXCR2 is essential for protective innate host response in murine *Pseudomonas aeruginosa* pneumonia, Infect. Immun. 68 (2000) 4289–4296.
[12] T. Van der Poll, C.V. Keogh, W.A. Buurman, S.F. Lowry, Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice, Am. J. Respir. Crit. Care Med. 155 (1997) 603–608.
[13] L.L. Laichalk, S.L. Kunkel, R.M. Strieter, J.M. Danforth, M.B. Bailie, T.J. Standiford, Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella pneumonia*, Infect. Immun. 64 (1996) 5211–5218.
[14] J.L. Flynn, J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, B.R. Bloom, An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection, J. Exp. Med. 178 (1993) 2249–2254.
[15] J.T. Harty, M.J. Bevan, Specific immunity to *Listeria monocytogenes* in the absence of IFN gamma, Immunity 3 (1995) 109–117.
[16] K. Kawakami, Y. Koguchi, M.H. Qureshi, A. Miyazato, S. Yara, Y. Kinjo, Y. Iwakura, K. Takeda, S. Akira, M. Kurimoto, A. Saito, IL-18 contributes to host resistance against infection with *Cryptococcus neoformans* in mice with defective IL-12 synthesis through induction of IFN-gamma production by NK cells, J. Immunol. 165 (2000) 941–947.

[17] Z.E. Wang, S.L. Reiner, S. Zheng, D.K. Dalton, R.M. Locksley, CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with *Leishmania major*, J. Exp. Med. 179 (1994) 1367–1371.
[18] K. Yoshida, T. Matsumoto, K. Tateda, K. Uchida, S. Tsujimoto, Y. Iwakurai, K. Yamaguchi, Protection against pulmonary infection with *Klebsiella pneumoniae* in mice by interferon-gamma through activation of phagocytic cells and stimulation of production of other cytokines, J. Med. Microbiol. 50 (2001) 959–964.
[19] X. Huang, S.A. McClellan, R.P. Barrett, L.D. Hazlett, IL-18 contributes to host resistance against infection with *Pseudomonas aeruginosa* through induction of IFN-gamma production, J. Immunol. 168 (2002) 5756–5763.
[20] N. Yamamoto, K. Kawakami, Y. Kinjo, K. Miyagi, T. Kinjo, K. Uezu, C. Nakasone, M. Nakamatsu, A. Saito, Essential role for the p40 subunit of interleukin-12 in neutrophil-mediated early host defense against pulmonary infection with *Streptococcus pneumoniae*: involvement of interferon-gamma, Microb. Infect. 6 (2004) 1241–1249.
[21] M. Kronenberg, L. Gapin, The unconventional lifestyle of NKT cells, Nat. Rev. Immunol. 2 (2002) 557–568.
[22] M. Taniguchi, M. Harada, S. Kojo, T. Nakayama, H. Wakao, The regulatory role of Valpha14 NKT cells in innate and acquired immune response, Annu. Rev. Immunol. 21 (2003) 483–513.
[23] M. Kronenberg, Toward an understanding of NKT cell biology: progress and paradoxes, Annu. Rev. Immunol. 23 (2004) 877–900.
[24] M. Skold, S.M. Behar, Role of CD1d-restricted NKT cells in microbial immunity, Infect. Immun. 71 (2003) 5447–5455.
[25] K. Kawakami, N. Yamamoto, Y. Kinjo, K. Miyagi, C. Nakasone, K. Uezu, T. Kinjo, T. Nakayama, M. Taniguchi, A. Saito, Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection, Eur. J. Immunol. 33 (2003) 3322–3330.
[26] K. Kawakami, Y. Kinjo, K. Uezu, S. Yara, K. Miyagi, Y. Koguchi, T. Nakayama, M. Taniguchi, A. Saito, Monocyte chemoattractant protein-1-dependent increase of Valpha 14 NKT cells in lungs and their roles in Th1 response and host defense in cryptococcal infection, J. Immunol. 167 (2001) 6525–6532.
[27] H. Ishikawa, H. Hisaeda, M. Taniguchi, T. Nakayama, T. Sakai, Y. Maekawa, Y. Nakano, M. Zhang, T. Zhang, M. Nishitani, M. Takashima, K. Himeno, CD4(+) Valpha14 NKT cells play a crucial role in an early stage of protective immunity against infection with *Leishmania major*, Int. Immunol. 12 (2000) 1267–1274.
[28] M.S. Duthie, M. Wleklinski-Lee, S. Smith, T. Nakayama, M. Taniguchi, S.J. Kahn, During *Trypanosoma cruzi* infection CD1d-restricted NK T cells limit parasitemia and augment the antibody response to alpha glycophosphoinositol-modified surface protein, Infect. Immun. 70 (2002) 36–48.
[29] B. Grubor-Bauk, A. Simmons, G. Mayrhofer, P.G. Speck, Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR, J. Immunol. 170 (2003) 1430–1434.
[30] Y. Kinjo, M. Kronenberg, Valpha14 *i*NKT cells are innate lymphocytes that participate in the immune response to diverse microbes, J. Clin. Immunol. 25 (2005) 522–533.
[31] T. Kawano, J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, H. Sato, E. Kondo, M. Harada, H. Koseki, T. Nakayama, Y. Tanaka, M. Taniguchi, Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5690–5693.
[32] Y. Tagawa, K. Sekikawa, Y. Iwakura, Suppression of concanavalin A-induced hepatitis in IFN-gamma−/−, but not in TNF-alpha−/− mice: role for IFN-gamma in activating apoptosis of hepatocytes, J. Immunol. 159 (1997) 1418–1428.
[33] H. Watanabe, K. Ohtsuka, M. Kimura, Y. Ikarashi, K. Ohmori, A. Kusumi, T. Ohteki, S. Seki, T. Abo, Details of an isolation method for hepatic lymphocytes in mice, J. Immunol. Methods 146 (1992) 145–154.
[34] H. Kitamura, K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. van Kaer, T. Kawano, M. Taniguchi, T. Nishimura, The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells, J. Exp. Med. 189 (1999) 1121–1128.
[35] E. Kobayashi, M. Motoki, T. Uchida, H. Fukushima, Y. Koezuka, KRN7000, a novel immunomodulator, and its antitumor activities, Oncol. Res. 7 (1995) 529–534.
[36] K. Kawakami, S. Kohno, N. Morikawa, J. Kadota, A. Saito, K. Hara, Activation of macrophages and expansion of specific T lymphocytes in the lungs of mice intratracheally inoculated with *Cryptococcus neoformans*, Clin. Exp. Immunol. 96 (1994) 230–237.
[37] G. Gonzalez-Aseguinolaza, C. de Oliveira, M. Tomaska, S. Hong, O. Bruna-Romero, T. Nakayama, M. Taniguchi, A. Bendelac, L. van Kaer, Y. Koezuka, M. Tsuji, alpha-galactosylceramide-activated Valpha 14 natural killer T cells mediate protection against murine malaria, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 8461–8466.
[38] K. Kawakami, Y. Kinjo, S. Yara, Y. Koguchi, K. Uezu, T. Nakayama, M. Taniguchi, A. Saito, Activation of Valpha14(+) natural killer T cells by alpha-galactosylceramide results in development of Th1 response and local host resistance in mice infected with *Cryptococcus neoformans*, Infect. Immun. 69 (2001) 213–220.
[39] K. Kakimi, L.G. Guidotti, Y. Koezuka, F.V. Chisari, Natural killer T cell activation inhibits hepatitis B virus replication in vivo, J. Exp. Med. 192 (2000) 921–930.
[40] N.Y. Crowe, M.J. Smyth, D.I. Godfrey, A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas, J. Exp. Med. 196 (2002) 119–127.
[41] R. Furlan, A. Bergami, D. Cantarella, E. Brambilla, M. Taniguchi, P. Dellabona, G. Casorati, G. Martino, Activation of invariant NKT cells by alphaGalCer administration protects mice from MOG35-55-induced EAE: critical roles for administration route and IFN-gamma, Eur. J. Immunol. 33 (2003) 1830–1838.
[42] H.Y. Kim, H.J. Kim, H.S. Min, S. Kim, W.S. Park, S.H. Park, D.H. Chung, NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor beta1 production, J. Exp. Med. 201 (2005) 41–47.
[43] F.N. Lauw, J. Branger, S. Florquin, P. Speelman, S.J. van Deventer, S. Akira, T. van der Poll, IL-18 improves the early antimicrobial host response to pneumococcal pneumonia, J. Immunol. 168 (2002) 372–378.
[44] T. Kinjo, M. Nakamatsu, C. Nakasone, N. Yamamoto, Y. Kinjo, K. Miyagi, K. Uezu, K. Nakamura, F. Higa, M. Tateyama, K. Takeda, T. Nakayama, M. Taniguchi, M. Kaku, J. Fujita, K. Kawakami, NKT cells play a limited role in the neutrophilic inflammatory response and host defense to pulmonary infection with *Pseudomonas aeruginosa*, Microb. Infect. 8 (2006) 2679–2685.
[45] M. Gonzalez-Juarrero, T.S. Shim, A. Kipnis, A.P. Junqueira-Kipnis, I.M. Orme, Dynamics of macrophage cell populations during murine pulmonary tuberculosis, J. Immunol. 171 (2003) 3128–3135.

International Immunology, Vol. 19, No. 6, pp. 755–762
doi:10.1093/intimm/dxm042
© The Japanese Society for Immunology. 2007. All rights reserved.
For permissions, please e-mail: journals.permissions@oxfordjournals.org

Schnurri-2 regulates T_h2-dependent airway inflammation and airway hyperresponsiveness

Chiaki Iwamura, Motoko Y. Kimura, Kenta Shinoda, Yusuke Endo, Akihiro Hasegawa, Masakatsu Yamashita and **Toshinori Nakayama**

Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Keywords: asthma, NF-κB, Schnurri-2-deficient mice

Abstract

Schnurri (Shn)-2 is a large zinc finger-containing protein, which plays a critical role in cell growth, signal transduction and lymphocyte development. In *Shn-2*-deficient (*Shn-2$^{-/-}$*) CD4 T cells, the activation of nuclear factor-κB is up-regulated and their ability to differentiate into T_h2 is enhanced. Here, we extend our investigation and demonstrate that Shn-2 regulates T_h2 responses *in vivo* using an ovalbumin-induced allergic asthma model. Eosinophilic inflammation, mucus hyperproduction and airway hyperresponsiveness (AHR) were all enhanced in *Shn-2$^{-/-}$* mice. Moreover, eosinophilic infiltration and AHR were enhanced in mice given a transfer of *Shn-2$^{-/-}$* effector T_h2. Shn-2 in T_h2 is thus considered to play an important role as a negative regulator in allergic airway inflammation.

Introduction

Drosophila Schnurri (Shn) is a large zinc-finger containing protein with a molecular weight of ~270 kDa. *Drosophila* Mad–Medea and Shn interact with each other and act as nuclear targets in the *Drosophila* decapentaplegic-signaling pathway (1–3). In vertebrates, this signaling pathway may equate to the bone morphogenetic protein/transforming growth factor-β/activin-signaling pathways which play diverse roles in the developmental processes. Vertebrates have at least three orthologs of Shn, namely Shn-1, Shn-2 and Shn-3 (4). mRNA expression of Shn-2 was detected primarily in the brain, heart and spleen. The vertebrate homologs of Shn were originally identified as proteins that bind to the nuclear factor-κB (NF-κB) site of various genes (5). Recently, the role of Shn-2 in the positive selection of thymocytes has been reported (6), and Shn-3-deficient $CD4^+CD8^+$ thymocytes were shown to exhibit a defect in cell survival (7). We recently demonstrated that Shn-2 binds to the NF-κB motif directly, thus resulting in the repression of the transcriptional activity of NF-κB through the competition of NF-κB binding in T cells (8). Shn-2-deficient (*Shn-2$^{-/-}$*) CD4 T cells showed an increased capability to differentiate into T_h2, due to the constitutive activation of NF-κB and the subsequent up-regulation of GATA3 expression (8). However, the precise physiological roles of these Shn family member proteins in *in vivo* immune responses still remain largely unknown.

T_h2 play an important role in allergic asthma by inducing allergen-specific IgE production, airway inflammation, airway hyperresponsiveness (AHR) and mucus hyperproduction (9–13). The administration of allergens adsorbed with alum induces reproducible allergen-specific acquired immune responses that are dependent on T_h2 producing IL-4, IL-5 and IL-13. A subsequent allergen challenge via the airway causes the rapid activation of T_h2, mast cells and B cells. This activation results in increased vascular permeability, cellular infiltration into the lung tissue, smooth muscle contraction and mucus secretion.

In this study, we investigated the role of Shn-2 in allergic inflammation using *Shn-2$^{-/-}$* mice. Our results suggest that Shn-2 plays a crucial role in the regulation of allergic airway inflammation and AHR.

Methods

Mice

Shn-2$^{-/-}$ mice have been described previously (6, 8). The animals used in this study were backcrossed to BALB/c >12 times and were 7–9 weeks old. Anti-ovalbumin (OVA)-specific TCRαβ (DO11.10) transgenic (Tg) mice were provided by Dennis Loh (Washington University School of Medicine, St Louis, MO, USA) (14). BALB/c mice were purchased from Clea Inc., Tokyo, Japan. Mice used in this study were at 7–9 weeks of age. Three independent experiments were performed for each experiment. All mice used in this study were maintained under specific pathogen-free conditions. All animal care was conducted in accordance with the guidelines of Chiba University.

Correspondence to: T. Nakayama; E-mail: tnakayama@faculty.chiba-u.jp
Transmitting editor: A. Singer

Received 23 November 2006, *accepted* 16 March 2007
Advance Access publication 9 May 2007

Sensitization and airway challenge with OVA

The mice were sensitized by an intra-peritoneal injection of 100 μg OVA (Sigma–Aldrich, St Louis, MO, USA) adsorbed to 1 mg alum (LSL, Tokyo, Japan) on day 0. OVA solution in PBS (100 μg per 30 μl) was administered intra-nasally to each mouse on days 7 and 9.

Measurement of AHR

The degree of AHR was assessed by methacholine-induced airflow obstruction 24 h after the last antigen challenge. The respiratory parameters were obtained by exposure of mice to 0.9% saline mist, followed by incremental doses of aerosolized methacholine (0, 3, 6, 12, 24 and 48 mg ml^{-1} in saline). Airflow obstruction was monitored and analyzed by whole-body plethysmograph (Buxco Electronics, Wilmington, NC, USA) as described previously (15). The results are expressed as the average in percentages of baseline enhanced paused values. The degree of AHR was also assessed by a computer-controlled small animal ventilator (SCIREQ, Montreal, Canada) (16). In brief, the mice were anesthetized with 100 μl per 10 g body weight of 50 mg ml^{-1} pentobarbital sodium given intra-peritoneally. After performing a tracheotomy, the trachea was cannulated with a blunted 18-gage needle. These mice were ventilated with a tidal volume of 10 ml kg^{-1} at a frequency of 180 breath min^{-1}. Each mouse was challenged with increasing doses of methacholine aerosol. After each challenge, lung resistance (RL) was recorded during tidal breathing every 10 s. The maximum values of RL were determined and expressed as the percent changes from baseline after saline exposure.

Collection of bronchioalveolar lavage fluid

Bronchioalveolar lavage (BAL) was performed 48 h after the last OVA challenge as described previously (17). All BAL fluid was collected and the cells were counted in 100-μl aliquots. One hundred thousand viable BAL cells were cytocentrifuged onto slides by a Cytospin 4 (Thermo Electron, Waltham, MA, USA) and stained with May-Grunwald–Giemza solution (MERCK, Darmstadt, Germany). Two hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The percentages of each cell type were calculated. Cytokine levels in the BAL fluid were measured 6 h after the last OVA challenges. IL-5, IL-13 and eotaxin-2 levels in BAL fluid were measured by ELISA as previously described (18).

Lung histology

The mice were sacrificed by asphyxiation at 48 h after the last OVA challenge, and the lungs were infused with 10% (v/v) formalin in PBS for fixation. The lung samples were sectioned, stained with hematoxylin and eosin (H&E) reagents or periodic acid–Schiff (PAS) reagent and examined for pathological changes under a light microscope at ×200. The number of infiltrated mononuclear cells in the peribronchiolar regions was calculated by direct counting in four different fields per slide.

Lung mononuclear cell preparation and a flow cytometry analysis

The lungs were sliced into small cubes and then incubated for 30 min in 5 ml RPMI 1640 solution containing collagenase (20 U ml^{-1}) (Worthington, Lakewood, NJ, USA) and trypsin inhibitor (0.3 mg ml^{-1}) (Sigma–Aldrich). Lung mononuclear cells were separated by centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK). For staining, one million cells were incubated on ice for 30 min with the appropriate staining reagents, according to a standard method (19). The reagents used in this study were anti-CD8α–PE (53-6.7) and anti-CD4–APC[K2] (RM4-5) purchased from PharMingen (San Diego, CA, USA). A flow cytometry analysis was performed on FACScalibur™[K3] (Becton Dickinson, Franklin lakes, NJ, USA) and the results were analyzed using the CELLQUEST™ software program (Becton Dickinson).

Adoptive cell transfer of T_h2 for the development of airway inflammation and AHR

Effector T_h2 were generated as previously described (20). In brief, splenic CD4 T cells purified from DO11.10 OVA-specific TCR Tg or *Shn-2*$^{-/-}$ DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 3 μM) plus antigen presenting cells under T_h2 culture conditions for 6 days *in vitro*. These effector T_h2 (5×10^{-6}) were transferred intravenously into BALB/c recipient mice on day 0. These recipient mice were not irradiated. On day 1 and 3, OVA solution (100 μg per 30 μl) was administrated intranasally to each mouse. The degree of AHR was measured on day 4. BAL fluid was collected on day 5.

Quantitative PCR analysis

Total RNA was isolated from the lung (three mice in each group) using the TRIzol reagent (Sigma–Aldrich). Reverse transcription (RT) was carried out with Superscript II RT (Invitrogen, Carlsbad, CA, USA). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under standard conditions. The primers and TaqMan probes for the detection of Muc5ac, thymus activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC) and hypoxanthine-guanine phosphoribosyltransferase (hprt) were purchased from Applied Biosystems. The expression of mRNA was normalized using the hprt signal.

Data analysis

The statistical analysis was performed using the two-tailed Student's *t*-test. Mann–Whitney *U*-tests were used to determine the level of difference in the degree of AHR. The values are the mean ± SD.

Results

Enhanced eosinophilic infiltration in BAL fluid and AHR in Shn-2$^{-/-}$ *mice*

We recently reported that the ability to differentiate into T_h2 *in vitro* was enhanced in *Shn-2*$^{-/-}$ naive CD4 T cells (8). The aim of this study was to clarify the role of Shn-2 in T_h2-dependent *in vivo* immune responses, such as OVA-induced allergic airway inflammation. Wild-type and *Shn-2*$^{-/-}$ mice were immunized with OVA–alum on day 0 and challenged with OVA intra-nasally on day 7 and 9. On day 11, BAL fluid was harvested and examined. The absolute numbers of eosinophils, lymphocytes, neutrophils and macrophages were determined by cell counts based on morphological criteria. As shown in Fig. 1(A), total cell numbers of infiltrating leukocyte significantly increased in the Shn-$2^{-/-}$ allergy-induced mice. A significant increase in the absolute number of eosinophils was also observed. In *Shn-2*$^{-/-}$ mice, both OVA immunization and OVA challenge were required for the induction of allergic inflammation. The levels of IL-5, IL-13 and eotaxin-2 were increased in allergy-induced *Shn-2*$^{-/-}$ mice in comparison to the levels in wild-type mice (Fig. 1B). No IL-4 was detected in the BAL fluid (data not shown).

We examined the degree of AHR in the allergy-induced *Shn-2*$^{-/-}$ mice by measuring methacholine-induced airflow obstruction with a whole-body plethysmograph (Fig. 1C) and a mechanical ventilator (Fig. 1D). The degree of AHR in *Shn-2*$^{-/-}$ mice was enhanced in comparison to that of wild-type mice. These data indicate that OVA-induced airway inflammation and AHR are therefore enhanced in allergy-induced *Shn-2*$^{-/-}$ mice.

Enhanced lung inflammation and mucus production in the lung of Shn-2$^{-/-}$ *mice*

We examined the histological changes in the lungs of allergy-induced *Shn-2*$^{-/-}$ mice by H&E staining (Fig. 2A, left panels). No massive inflammatory cell infiltration was noted in the lungs of wild-type and *Shn-2*$^{-/-}$ mice that did not receive the OVA challenge (Fig. 2A, panels a and c). Substantial numbers of mononuclear cells were infiltrated in the peribronchiolar regions in wild-type mice after the OVA challenge (Fig. 2A, panel b), and the infiltration extended to the surrounding area in *Shn-2*$^{-/-}$ mice (Fig. 2A, panel d). The number of infiltrated cells also increased in *Shn-2*$^{-/-}$ mice (Fig. 2A, right panels).

We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in allergy-induced *Shn-2*$^{-/-}$ mice are shown (Fig. 2B). No specific staining was detected in wild-type and *Shn-2*$^{-/-}$ mice without the OVA challenge (Fig. 2B, panels a and c). Moderate staining was noted in wild-type bronchioles, whereas the staining levels increased in the

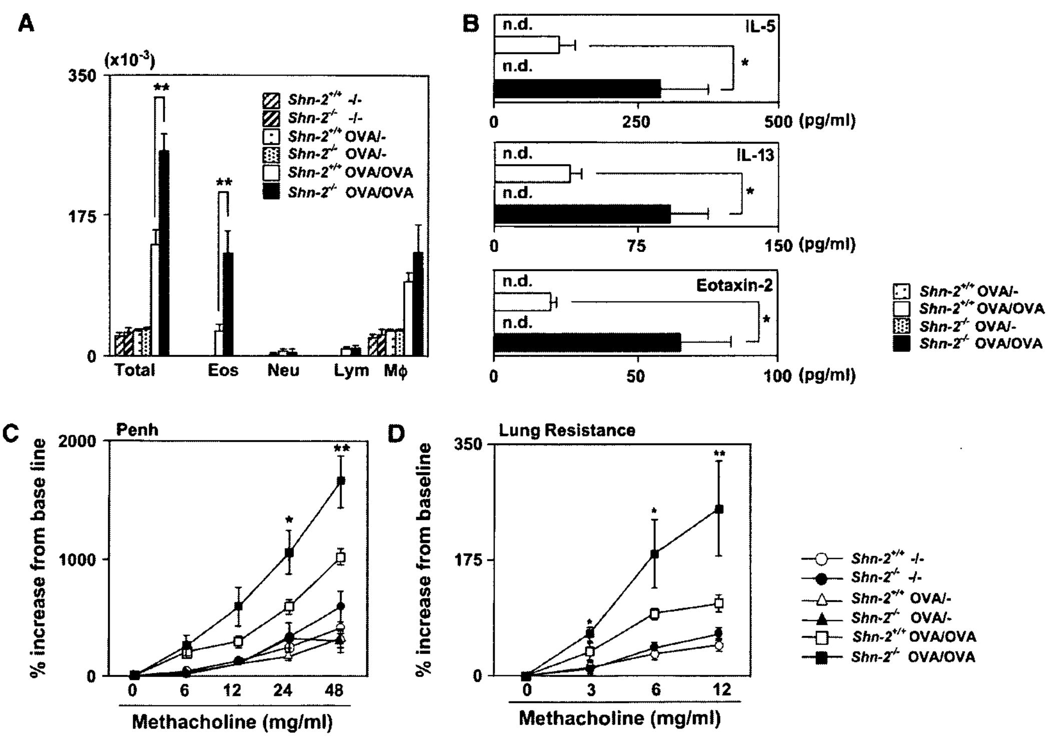

Fig. 1. Enhanced airway inflammation and AHR in *Shn-2*$^{-/-}$ mice. Airway inflammation and AHR were induced with OVA sensitization and challenges. (A) The absolute numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mϕ) in the BAL fluid are shown. The results were obtained using the values from cell counting, the percentages of the cells, total cell number per milliliter and the volume of BAL fluid recovered. Samples ware collected 48 h after the last OVA challenge. Mean values with SDs (n = 5) are shown. Four independent experiments were done with similar results. –/–: without OVA priming or OVA challenge, OVA/–: with OVA priming but not OVA challenge and OVA/OVA: with OVA priming and OVA challenge. (B) The levels of IL-5, IL-13 and eotaxin-2 in BAL fluid were determined by ELISA. Samples were collected 6 h after the last OVA challenge. Mean values with SDs (n = 5) are shown. (C and D) One day after the last OVA challenge, AHR in response to increasing doses of methacholine was assessed by measuring enhanced pause (C) and RL (D). Five animals from each group were individually examined, and the mean values and SDs are indicated. Four independent experiments were done with similar results. The differences were statistically significant between wild-type and *Shn-2*$^{-/-}$ mice with OVA sensitization and OVA challenge (*P < 0.05 and **P < 0.01).

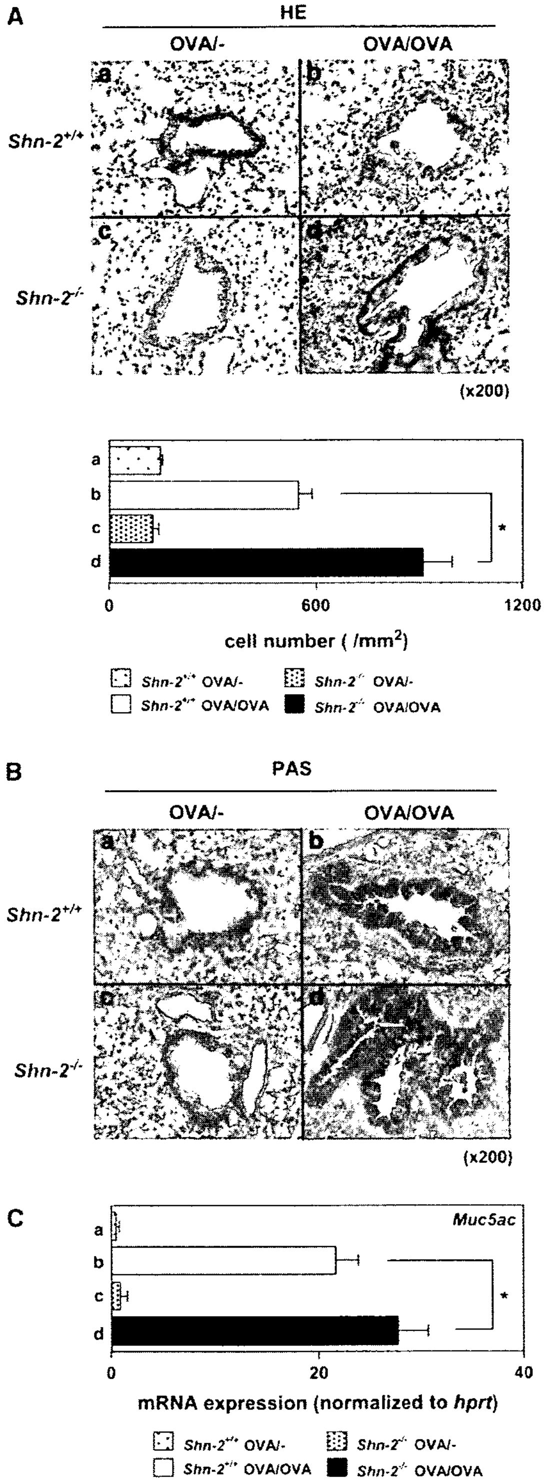

Fig. 2. Enhanced leukocyte infiltration into the lung and mucus production in allergy-induced *Shn-2*$^{-/-}$ mice. The levels of OVA-induced airway inflammation and mucus production in *Shn-2*$^{-/-}$ mice were examined by a histological analysis and a quantitative real-time RT-PCR. (A) Antigen-induced leukocyte infiltration into the lung was evaluated using H&E staining (left panels). The numbers of infiltrated mononuclear cells in the perivascular and peribronchiolar regions were calculated by direct counting from four different fields per slide (right panels). The mean values with SDs (n = 5) are shown (*P < 0.05). (B) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photographic views of wild-type and *Shn-2*$^{-/-}$ mice are shown. (C) Total mRNA was prepared from the lung of allergy-induced wild-type or *Shn-2*$^{-/-}$ mice, and mRNA levels of Muc5ac were examined. The data represent the mean values of Muc5ac mRNA expression normalized with *hprt* expression. Three independent experiments were done with similar results (*P < 0.05).

Shn-2$^{-/-}$ bronchioles (Fig. 2B, panels b and d). Consequently, we examined the expression of Muc5ac in the lungs of *Shn-2*$^{-/-}$ mice, and a slight, but significant increase in the expression was noted in the *Shn-2*$^{-/-}$ mouse lungs (Fig. 2C). These results indicate that the levels of mucus hyperproduction were moderately enhanced in the lungs of the allergy-induced *Shn-2*$^{-/-}$ mice in comparison to those of the wild-type mice.

The increased number of lung CD4 T cells in Shn-2$^{-/-}$ *mice is accompanied with an enhanced production of TARC and MDC*

Previous studies have reported a reduced number of CD4 T cells in the spleen of *Shn-2*$^{-/-}$ mice (8). We therefore examined whether the number of CD4 T cells were reduced in OVA-sensitized *Shn-2*$^{-/-}$ mice. Lung leukocytes were stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. The percentages of CD4^{+} and CD8^{+} cells in *Shn-2*$^{-/-}$ mice significantly decreased in comparison to those in wild-type mice (Fig. 3A). After the OVA challenge, the percentage of CD4 T cells increased substantially in the *Shn-2*$^{-/-}$ mice (12.1 versus 23.4%). The absolute numbers of total leukocytes and CD4 T cells in the lung decreased significantly in *Shn-2*$^{-/-}$ mice (Fig. 3B, upper panels). However, a dramatic increase in the total numbers of lung leukocytes and CD4 T cells was observed in the *Shn-2*$^{-/-}$ mice after the OVA challenge (Fig. 3B, lower panels).

In our previous study, the proliferative ability of CD4 T cells in the *Shn-2*$^{-/-}$ mice was comparable to that in the wild-type mice (8). To investigate the reason why the CD4 T cell levels increased in the allergy-induced *Shn-2*$^{-/-}$ mice, we examined the mRNA expression of TARC and MDC. These chemokines are known to be selective attractants for T_h2 migration (21). As shown in Fig. 3(C), the mRNA expression levels of TARC and MDC in the lung from the *Shn-2*$^{-/-}$ mice were significantly higher than those in the wild-type mice. The increased expression of TARC and MDC may thus explain the dramatic increase observed in the number of CD4 T cells in the lungs of *Shn-2*$^{-/-}$ mice.

Shn-2$^{-/-}$ *effector* T_h2 *enhanced AHR and eosinophilic infiltration into the lungs in recipient mice*

We performed adoptive transfer experiments to determine whether the enhancement of airway inflammation and AHR observed in *Shn-2*$^{-/-}$ mice is mediated via *Shn-2*$^{-/-}$ T_h2. Effector T_h2 from *Shn-2*$^{-/-}$ DO11.10 OVA-specific TCR Tg mice were prepared as described in the Methods. On days

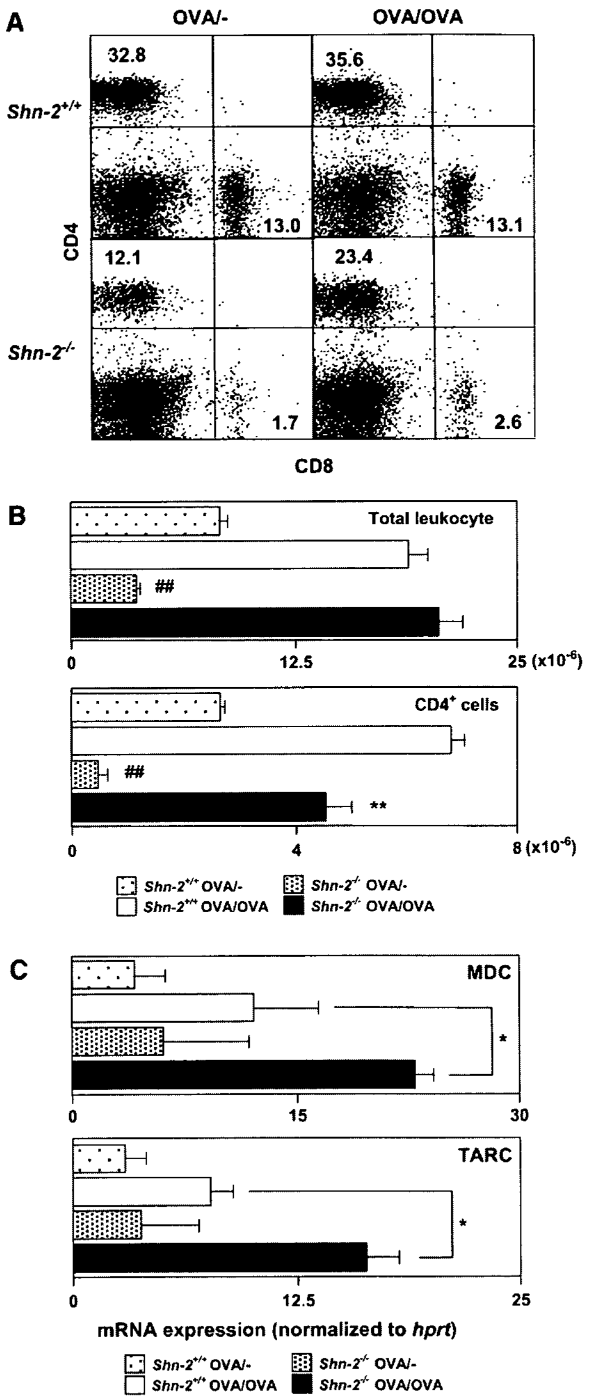

Fig. 3. Increased CD4 T cells in the lung tissues after OVA challenge in *Shn-2*$^{-/-}$ mice. Lung mononuclear cells from *Shn-2*$^{-/-}$ mice were prepared as described in the Methods. (A) Representative CD4/CD8 profiles of the lung leukocytes from wild-type or *Shn-2*$^{-/-}$ mice before (OVA/−) and after OVA challenge (OVA/OVA). The percentages of cells in each quadrant are shown. (B) Total numbers of leukocytes harvested in the lung (upper panels) and the number of CD4 T cells (lower panels) are shown. Differences in the number of leukocytes and CD4 T cells were statistically significant between the wild-type and the *Shn-2*$^{-/-}$ mice without OVA challenge ($^{\#\#}P < 0.01$). The differences in the number of CD4 T cells were statistically significant between the wild-type mice and the *Shn-2*$^{-/-}$ mice with OVA sensitization and OVA challenge ($^{**}P < 0.01$). (C) Total mRNA was prepared from the lung of allergy-induced wild-type or *Shn-2*$^{-/-}$ mice. A real-time RT-PCR analysis for TARC and MDC as well as *hprt* (as a control) was performed. Representative data of three individual animals from three independent experiments are shown ($^{*}P < 0.05$).

1 and 3 after effector T_h2 transfer into syngeneic BALB/c mice, airway inflammation was induced by the OVA intranasal administration. A significant increase in the absolute number of eosinophilic infiltration in BAL fluid was observed in the mice that received *Shn-2*$^{-/-}$ T_h2 (Fig. 4A). The levels of IL-5 and eotaxin-2 in the BAL fluid from the mice receiving *Shn-2*$^{-/-}$ T_h2 increased markedly more than that from the mice receiving wild-type T_h2 (Fig. 4B). The levels of IL-4 were not increased but moderately decreased. The levels of IL-13 were comparable. The degree of AHR in the mice receiving *Shn-2*$^{-/-}$ T_h2 also increased more than that in the mice receiving wild-type T_h2 (Fig. 4C and D). These results suggest that the hyperactivation of effector T_h2 in *Shn-2*$^{-/-}$ mice exacerbates the development of allergic airway inflammation.

We then examined the levels of mucus hyperproduction by PAS staining. Representative staining profiles of the bronchiolar regions in the mice that received wild-type or *Shn-2*$^{-/-}$ T_h2 are shown (Fig. 4E). Moderate staining was noted in both bronchioles (Fig. 4E, panels a and b). The mRNA expression of Muc5ac in the lung of mice that received *Shn-2*$^{-/-}$ T_h2 was examined, and it was found to be comparable to that in the mice receiving wild-type T_h2 (Fig. 4F). These results indicate that the levels of mucus hyperproduction in the lungs of the mice that received *Shn-2*$^{-/-}$ T_h2 are therefore comparable to those of the mice that received wild-type T_h2.

Discussion

We previously reported that the activation of NF-κB to be up-regulated in *Shn-2*$^{-/-}$ CD4 T cells, and their ability to differentiate into T_h2 was enhanced (8). In this study, we demonstrated that OVA-induced allergic inflammation and AHR are enhanced in the *Shn-2*$^{-/-}$ mice as well as in the wild-type mice transferred with *Shn-2*$^{-/-}$ effector T_h2. These results indicate that Shn-2 regulates OVA-induced airway inflammation and AHR through the control of CD4 T cell activation.

We observed an increased IL-5, IL-13 and eotaxin-2 level in the BAL fluid in OVA-sensitized and OVA-challenged *Shn-2*$^{-/-}$ mice (Fig. 1B). IL-13 is known to induce AHR in the absence of inflammatory cells (13). IL-5 and eotaxin-2 are known to attract eosinophils (22). Since eosinophils release granule proteins that are cytotoxic to the airway epithelium such as major basic proteins, eosinophilia may exacerbate the airway obstruction and AHR (23). Therefore, it is likely that the overproduction of these factors (IL-5, IL-13 and eotaxin-2) resulted in the enhanced eosinophilic infiltration and AHR in the airways of the *Shn-2*$^{-/-}$ mice. The hyperproduction of mucus also plays an important role in the pathogenesis of various asthmatic features and is linked with

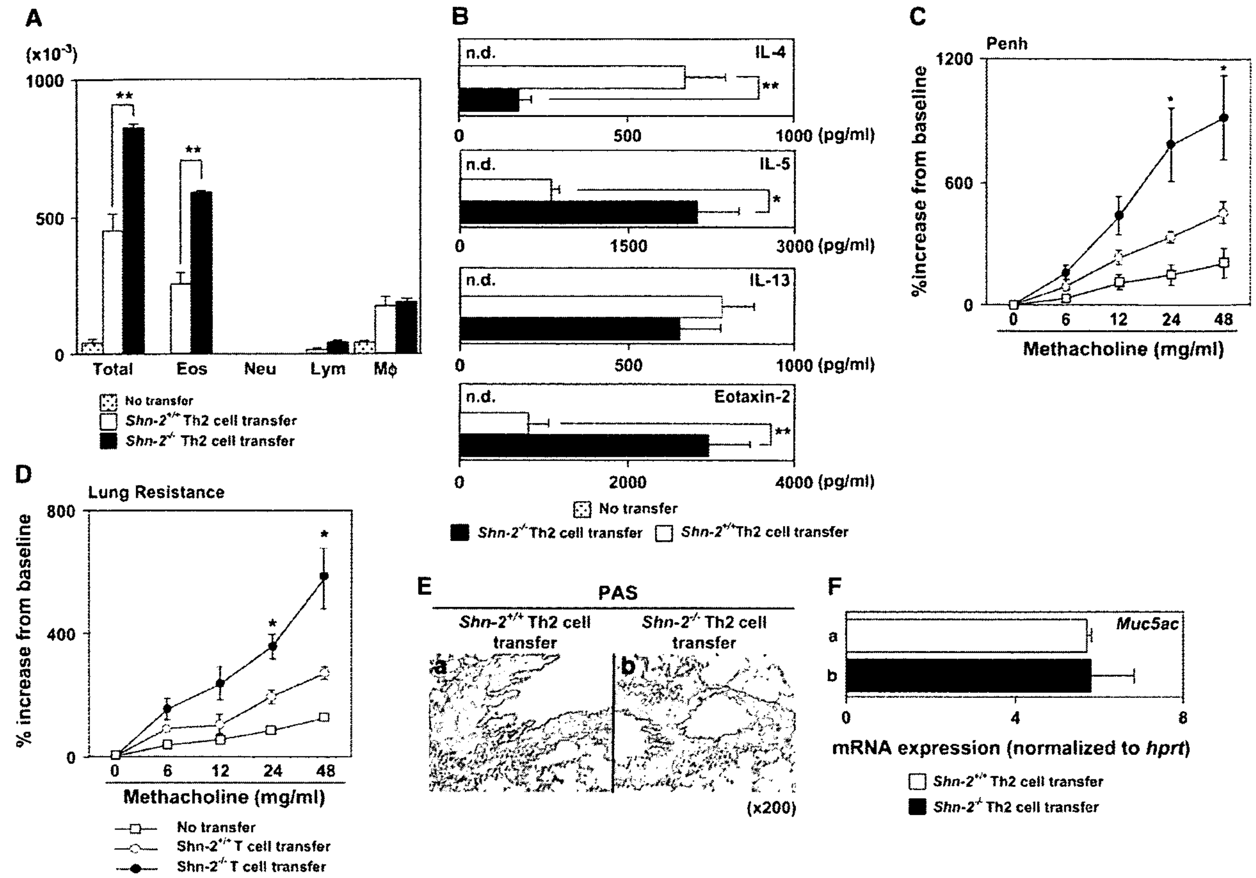

Fig. 4. Enhanced airway inflammation and AHR in mice receiving *Shn-2*$^{-/-}$ effector T_h2. Naive CD4 T cells from DO11.10 OVA-specific TCR Tg or *Shn-2*$^{-/-}$ DO11.10 OVA-specific TCR Tg mice were cultured under T_h2 conditions for 6 days. The effector T_h2 (5×10^6) were transferred into BALB/c mice 1 day before the first OVA challenge. (A) The absolute number of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym) and macrophages (Mϕ) in the BAL fluid are shown. The mean values with SDs ($n = 5$) are shown. Three independent experiments were done with similar results (**$P < 0.01$). (B) The levels of IL-4, IL-5, IL-13 and eotaxin-2 in the BAL fluid were determined by ELISA. Samples ware collected 6 h after the last OVA challenge. The mean values with SDs ($n = 5$) are shown. Three independent experiments were done with similar results (*$P < 0.05$ and **$P < 0.01$). n.d. not detectable. (C and D) AHR was monitored by measuring enhanced pause (left panel) and RL (right panel) as described in the Methods. The mean values with SDs ($n = 5$) are shown. (E) Antigen-induced goblet cell hyperplasia was evaluated by PAS staining. Representative photographic views of the lung in the mice receiving wild-type or *Shn-2*$^{-/-}$ T_h2 are shown. (F) The data represent the mean values of Muc5ac mRNA expression in the lung of mice receiving wild-type or *Shn-2*$^{-/-}$ T_h2. It was normalized with *hprt* expression. Three independent experiments were done with similar results. Differences in AHR were statistically significant between wild-type and *Shn-2*$^{-/-}$ T cell transfer groups (*$P < 0.05$).

asthma fatality (24, 25). IL-13 has been shown to induce mucus hypersecretion *in vivo* and *in vitro* (26). Therefore, an overproduction of IL-13 may induce severe mucus secretion in the *Shn-2*$^{-/-}$ lung. IL-13 was also shown to induce TARC and MDC production from keratinocytes and bronchial epithelial cells (27, 28). The increased number of CD4 T cells in the lungs of the *Shn-2*$^{-/-}$ mice may be due to the increased amount of IL-13, which thus resulted in the overproduction of TARC and MDC.

To investigate whether the exacerbation of airway inflammation and AHR was due to the deficiency of Shn-2 in T_h2, we performed a set of experiments with adoptive transfer of T_h2 (Fig. 4). As a result, an enhanced degree of eosinophilic infiltration and increased levels of IL-5 and eotaxin-2 in BAL fluid were observed in wild-type recipient mice transferred with *Shn-2*$^{-/-}$ T_h2. The degree of AHR was also amplified. Therefore, the exacerbation of airway inflammation and AHR appears to be at least in part due to the enhanced T_h2 activities of *Shn-2*$^{-/-}$ T_h2.

However, the levels of IL-13 in the BAL fluid from the mice that received *Shn-2*$^{-/-}$ T_h2 were comparable to those from the mice that received wild-type T_h2. The levels of mucus hypersecretion did not increase in the mice that received *Shn-2*$^{-/-}$ T_h2 (Fig. 4E and F). These results may indicate that the overproduction of IL-13 in the BAL fluid of *Shn-2*$^{-/-}$ mice was not only from CD4 T cells but also from other IL-13-producing cells such as mast cells, basophils and eosinophils. We observed the Shn-2 expression in naive CD4 T cells as well as bone marrow-derived mast cells (BMMCs) (Supplementary Figure 1A, available at *International Immunology*

Online). The phenotypic features (expression profiles of c-kit and FcεRI) were indistinguishable between wild-type and *Shn-2*$^{-/-}$ BMMCs (Supplementary Figure 1B, available at *International Immunology* Online.). In addition, the levels of IL-5, IL-6 and IL-13 production after cross-linking of FcεRI using anti-DNP IgE and DNP-BSA were comparable (Supplementary Figure 1C, available at *International Immunology* Online.). The IL-4 production was not detected by ELISA in either group (C. Iwamura and T. Nakayama, unpublished observation). These results indicate that the function of *Shn-2*$^{-/-}$ mast cells is thus within the normal range.

We observed that the production of T_h2-dependent antibodies (IgG1 and IgE) induced by OVA–alum immunization was decreased in *Shn-2*$^{-/-}$ mice as compared with that seen in wild-type mice (M. Y. Kimura and T. Nakayama, unpublished observation). The level of IL-4 in the BAL fluid in the mice that received *Shn-2*$^{-/-}$ T_h2 decreased (Fig. 4B). This could be the reason why the IgG1 and IgE levels are decreased in the *Shn-2*$^{-/-}$mice. It is unclear at this time why the production of IL-4 in the BAL fluid of mice that received *Shn-2*$^{-/-}$ T_h2 decreased. One possible explanation is the fact that GATA3 may play a more important role in the regulation of IL-5 production than IL-4.

In addition to T_h2, other lymphocytes substantially regulate allergic diseases. It has been reported that CD8 T cells and NKT cells are the source of IL-13 and can induce the airway inflammation and AHR independently from conventional CD4 T cells (29–31). Regulatory T cells are also known to control allergic diseases (12, 32). In *Shn-2*$^{-/-}$ mice, the absolute number of $CD4^{+}CD25^{+}$ T cells decreased in comparison to that of wild-type mice (C. Iwamura and T. Nakayama, unpublished observation). Furthermore, some *Shn-2*$^{-/-}$ mice after 16 weeks of age died from severe whole-body inflammation of an enlarged spleen and draining lymph nodes (C. Iwamura and T. Nakayama, unpublished observation). Therefore the down-regulation of regulatory T cells may exacerbate the airway inflammation in *Shn-2*$^{-/-}$ mice. Not only for T cells but also for non-T cell populations, such as airway smooth muscle cells, eosinophils and epithelial cells, which have all been reported to play important roles in the development of asthma (9, 22, 33). Although we need to await a more comprehensive study, it is possible that some of these cells may express Shn-2, and thereby contribute to the exacerbation of airway inflammation and AHR in *Shn-2*$^{-/-}$ mice.

Recently, the role of NF-κB in the pathogenesis of allergic diseases was investigated in experimental allergic murine models (34–36). Since *Shn-2*$^{-/-}$ CD4 T cells showed the constitutive activation of NF-κB (8), we used an adoptive transfer system to examine whether or not the effector T_h2 with an increased activation level of NF-κB exacerbate airway inflammation and AHR. Our preliminary results showed significant increases in the absolute number of eosinophils and the degree of AHR was observed in the mice that received T_h2 over-expressing NF-κB (p65) (C. Iwamura and T. Nakayama, unpublished observation). These data suggest that the increased levels of NF-κB activation in *Shn-2*$^{-/-}$ T_h2 may thus enhance both airway inflammation and AHR.

In summary, OVA-induced eosinophilic airway inflammation, AHR and mucus hyperproduction were all found to be enhanced in *Shn-2*$^{-/-}$ mice. Therefore, Shn-2 appears to play a key role as an *in vivo* negative regulator of the T_h2-dependent allergic airway responses.

Supplementary data

Supplementary Figure 1 is available at *International Immunology* Online.

Acknowledgements

We are grateful to Erwin Gelfand for the introduction of the measurement of RL and to Yoichi Suzuki for his valuable advice on the statistical analysis. We also thank Kaoru Sugaya, Hikari Asou, Satoko Norikane and Toshihiro Ito for their excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid for Scientific Research in Priority Areas #17016010 and #17047007, Scientific Research B #17390139, Scientific Research C #18590466; Grant-in-Aid for Young Scientists #17790318; Special Coordination Funds for Promoting Science and Technology), the Ministry of Health, Labor and Welfare (Japan), The Japan Health Science Foundation, Kanae Foundation, Uehara Memorial Foundation, Mochida Foundation and Sagawa Foundation.

Abbreviations

AHR	airway hyperresponsiveness
BAL	bronchioalveolar lavage
BMMC	bone marrow-derived mast cell
H&E	hematoxylin and eosin
hprt	hypoxanthine guanine phosphoribosyl transferase
MDC	macrophage-derived chemokine
NF-κB	nuclear factor-κB
OVA	ovalbumin
PAS	periodic acid–Schiff
RL	lung resistance
RT	reverse transcription
Shn	Schnurri
Shn-2$^{-/-}$	Shn-2 deficient
TARC	thymus activation-regulated chemokine
Tg	transgenic

References

1 Affolter, M., Marty, T., Vigano, M. A. and Jazwinska, A. 2001. Nuclear interpretation of Dpp signaling in *Drosophila*. *EMBO J.* 20:3298.

2 Arora, K., Dai, H., Kazuko, S. G. *et al.* 1995. The *Drosophila* schnurri gene acts in the Dpp/TGF beta signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell* 81:781.

3 Staehling-Hampton, K., Laughon, A. S. and Hoffmann, F. M. 1995. A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for dpp signaling. *Development* 121:3393.

4 Wu, L. C. 2002. ZAS: C2H2 zinc finger proteins involved in growth and development. *Gene Expr.* 10:137.

5 Maekawa, T., Sakura, H., Sudo, T. and Ishii, S. 1989. Putative metal finger structure of the human immunodeficiency virus type 1 enhancer binding protein HIV-EP1. *J. Biol. Chem.* 264: 14591.

6 Takagi, T., Harada, J. and Ishii, S. 2001. Murine Schnurri-2 is required for positive selection of thymocytes. *Nat. Immunol.* 2:1048.

7 Allen, C. E., Muthusamy, N., Weisbrode, S. E., Hong, J. W. and Wu, L. C. 2002. Developmental anomalies and neoplasia in animals and cells deficient in the large zinc finger protein KRC. *Genes Chromosomes Cancer* 35:287.

8 Kimura, M. Y., Hosokawa, H., Yamashita, M. *et al.* 2005. Regulation of T helper type 2 cell differentiation by murine Schnurri-2. *J. Exp. Med.* 201:397.