hph DNA at this time-point were virtually superimposable with the relative levels of infected cells (dashed lines). These results suggest that a uniform fraction – perhaps all or nearly all – of the hph DNA molecules that were stably associated with the infected cells were present in functional, integrated proviruses.

It seemed possible that the hph DNA molecules that are not stably associated with the cells could be blocked with respect to entry into the nucleus, prior to proviral integration. The presence of closed 2-LTR circular DNA is frequently taken as an indicator of entry of viral DNA into the nucleus (e.g., Yuan et al., 2002). We therefore measured the level of 2-LTR circles in the cells 24 h after infection. As shown in Fig. 6 (triangles), the relative levels of these circles are significantly higher than the levels of infection or of stable hph DNA and correspond closely with the level of hph DNA in the 24-h lysates. Thus, there is no indication that the nuclear entry of DNA molecules is blocked in the cells infected with the mixed virions.

Discussion

We have shown here (Fig. 4) that two MLV Gag proteins which lack the absolutely conserved proline residue at the N-terminus of the capsid domain are potent dominant-negative inhibitors of infectivity when present in virions together with the wild-type protein. In one of these, S2D, the change in the Gag coding sequence prevents cleavage at the p12-CA boundary, so that no free N-terminus of CA is formed during virus maturation. In contrast, the other mutant studied, S2G, undergoes efficient cleavage at this site, but the resulting N-terminal residue is glycine rather than proline. (Cleavage at the C-terminus of CA is, however, incomplete in S2G mutant particles (Fig. 1B). It is somewhat surprising that the cleavage at site 3 is inhibited by the replacement of proline with glycine at site 2).

We attempted to identify the point in the viral replication cycle that is blocked in the particles containing both wild-type and mutant Gag proteins. We found (Fig. 6) that the proportion of mixed particles synthesizing late (i.e., hph) DNA products is reduced in the mixed particles but is still significantly higher than the proportion that successfully establishes infection. The presence of the mutant Gag proteins also has a less drastic effect on the synthesis of 2-LTR circular DNA than on infection or stable association of viral DNA with the cells, presumably in integrated proviruses. Taken together, these results imply that there is a fraction of the phenotypically mixed virions which synthesizes DNA, and that this DNA enters the nucleus but is not integrated into cellular DNA. One hypothesis that could explain these results is that these DNA molecules are unsuitable substrates for integrase: perhaps there are subtle defects in their ends, as has been previously described for mutants in NC (Gorelick et al., 1999) and, in HIV-1, in the RNase H domain of reverse transcriptase (Julias et al., 2002).

The ability of the mutant proteins to interfere with the function of the wild-type CA in the mixed particles implies that wild-type proteins do not function as monomers, but as part of a large, organized assemblage; this large functional unit is apparently disrupted and inactivated by the presence of mutant proteins within it. This structure is presumably the mature core of the virion. Mutants elsewhere within HIV-1 CA have previously been shown to interfere with infectivity in mixed particles (Furuta et al., 1997; Mammano et al., 1994; Trono et al., 1989). Interestingly, a recent study of poliovirus pointed out that genes in which mutations show trans-dominant effects can be particularly attractive targets for antiviral therapy; indeed, screening for trans-dominance can be used in the selection of drug targets (Crowder and Kirkegaard, 2005). Taken together, these observations highlight the potential utility of particle assembly and maturation as potential antiretroviral drug targets. Unfortunately, the lack of basic molecular understanding and of convenient screens has made it impossible to exploit these targets to date.

The results presented here have, in part, been foreshadowed by previous reports using low doses of PR inhibitors on HIV-1. It was found in these studies that drug concentrations which only prevent cleavage in a small fraction of the Gag and Pol proteins lead to production of noninfectious particles (Kaplan et al., 1993; Krausslich, 1992). It was also reported that the loss of infectivity in these particles is more severe than the defect in reverse transcription (Kaplan et al., 1993). Thus, these results, like those in the present study, imply that a minority of uncleaved viral proteins (as in the S2D:wild-type mixed particles) can block infectivity without blocking DNA synthesis. However, they do not provide information as to which cleavage sites are so critical for infection.

The present results also add to the growing body of evidence indicating that the mature core of a retrovirus particle plays a crucial role in reverse transcription. Thus, mutations in HIV-1 CA that alter the stability of the core in viral lysates affect the kinetics of DNA synthesis during infection (Forshey et al., 2002). In addition, mutations that replace either the N-terminal proline or its aspartate partner in the buried salt bridge in CA interfere with both formation of the normal core morphology and the initiation of viral DNA synthesis upon infection (Fitzon et al., 2000; Tang et al., 2003; von Schwedler et al., 1998). These studies all suggest that uncoating of the viral genome is a critical, tightly regulated step in infection which requires the correct conformation of the N-terminus of CA. In MLV also, mutants near the N-terminus of CA have been shown to be impaired in reverse transcription (Auerbach et al., 2003). However, we are not aware of prior reports implicating CA in the successful completion of integration-competent viral DNA copies, as suggested by the data in Fig. 6. In this connection, it is interesting to note that retroviral CA proteins are also the target of several cellular restriction systems, including Fv-1, Lv-1, and Ref-1 (Lee and KewalRamani, 2004). It appears that some of these systems interfere with infection before viral DNA synthesis (Stremlau et al., 2004; Towers et al., 2000), while others interfere with a subsequent event (Jolicoeur and Rassart, 1980; Yang et al., 1980); again, these observations would suggest that CA functions both in the initiation of reverse transcription and in its successful completion or in the integration of the DNA product.

Materials and methods

Cells and viruses

All experiments described here were performed with full-length Moloney MLV proviral clones ultimately derived from the infectious clone pRR88 (Fu and Rein, 1993). Virus particles were produced by transient transfection of 293T cells, using Transit 293 (Mirrus) in accord with the manufacturer's instructions. When viruses were to be analyzed for infectivity, either pBABE-Luc, which contains the firefly luciferase gene from pGL3 (Promega) in pBabe Puro (Morgenstern and Land, 1990), or pLZRS-EGFP, an MLV-based vector encoding GFP (Dardalhon et al., 1999), was co-transfected with the MLV proviral constructs being analyzed. Infectivity was then measured by infecting either NIH3T3 cells or 293T cells expressing the ecotropic MLV receptor MCAT (Albritton et al., 1989) (a kind gift of J. Cunningham), and either enumerating GFP-positive cells as described (Oshima et al., 2004) or assaying cell extracts for luciferase activity using the Luciferase Assay System (Promega) 48 h after infection. The cell extracts were also assayed for total protein content using the BCA assay system (Pierce) according to the manufacturer's instructions. MLV containing a mutation at the active site of reverse transcriptase, changing residues 223 and 224 from aspartate to lysine and leucine, respectively (a kind gift from Robert Gorelick, SAIC Frederick), was used as a negative control. Except where specified otherwise, all techniques were as described (Oshima et al., 2004).

Electron microscopy

Released virus was examined after it was collected by immunoprecipitation on protein G-Sepharose beads as previously described (Campbell et al., 2002).

Viral RNA

Virions were assayed for hph RNAby real-time RT-PCR as described (Hibbert et al., 2004).

Viral DNA synthesis

The ability of virus particles to perform DNA synthesis upon infecting new host cells was assayed as follows. Viruses were produced by transient transfection of 293T cells that had previously been stably transfected with pLXSH (Miller et al., 1993) and selected for hygromycin resistance. The virus particles obtained following the transient transfection were then used to infect 293T cells expressing MCAT. 24 h later, the cells were lysed by the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and the cell extracts were assayed for hygromycin phosphotransferase (hph) DNA by real-time PCR as described (Hibbert et al., 2004). Two-LTR circles were assayed by real-time PCR using 5′-GTC TCG CTG TTC CTT GGG AGG GTC-3′ as the forward primer, 5′-CAT CTG TTC CTG ACC TTG ATC TGA-3′ as the reverse primer, and 5′ FAM-GGC GTT ACT TAA GCT AGC TTG CCA–TAMRA-3′ as the probe. These reagents were designed to anneal to sequences found in both MLV and pLXSH. The extracts were also assayed for the single-copy gene CCR5 by real-time PCR in order to control for differences in recovery of DNA from the extracts; these assays used 5′-CCA GAA GAG CTG AGA CAT CCG-3′ for forward primer, 5′-GCC AAG CAG CTG AGA GGT TAC T-3′ for reverse primer, and 5′FAM-TCCCCTA-CAAGAAACTCTCCCCGG-TAMRA-3′ for probe. Plasmids used as standards in these two assays were a kind gift of Robert Gorelick.

Acknowledgments

We thank Vineet KewalRamani, Zandrea Ambrose, Robert J. Gorelick, James Thomas and James Cunningham for reagents and for many helpful discussions. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and was funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-12400.

References

Albritton, L.M., Tseng, L., Scadden, D., Cunningham, J.M., 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57 (4), 659–666.

Auerbach, M.R., Shu, C., Kaplan, A., Singh, I.R., 2003. Functional characterization of a portion of the Moloney murine leukemia virus gag gene by genetic footprinting. Proc. Natl. Acad. Sci. 100 (20), 11678–11683.

Campbell, S., Oshima, M., Mirro, J., Nagashima, K., Rein, A., 2002. Reversal by dithiothreitol treatment of the block in murine leukemia virus maturation induced by disulfide cross-linking. J. Virol. 76 (19), 10050–10055.

Crowder, S., Kirkegaard, K., 2005. Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. Nat. Genet. 37 (7), 701–709.

Dardalhon, V., Noraz, N., Pollok, K., Rebouissou, C., Boyer, M., Bakker, A.Q., Spits, H., Taylor, N., 1999. Green fluorescent protein as a selectable marker of fibronectin-facilitated retroviral gene transfer in primary human T lymphocytes. Hum. Gene Ther. 10 (1), 5–14.

Fitzon, T., Leschonsky, B., Bieler, K., Paulus, C., Schroder, J., Wolf, H., Wagner, R., 2000. Proline residues in the HIV-1 NH2-terminal capsid domain: structure determinants for proper core assembly and subsequent steps of early replication. Virology 268 (2), 294–307.

Forshey, B.M., von Schwedler, U., Sundquist, W.I., Aiken, C., 2002. Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. J. Virol. 76 (11), 5667–5677.

Fu, W., Rein, A., 1993. Maturation of dimeric viral RNA of Moloney murine leukemia virus. J. Virol. 67 (9), 5443–5449.

Furuta, R.A., Shimano, R., Ogasawara, T., Inubushi, R., Amano, K., Akari, H., Hatanaka, M., Kawamura, M., Adachi, A., 1997. HIV-1 capsid mutants inhibit the replication of wild-type virus at both early and late infection phases. FEBS Lett. 415 (2), 231–234.

Gamble, T.R., Vajdos, F.F., Yoo, S., Worthylake, D.K., Houseweart, M., Sundquist, W.I., Hill, C.P., 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell 87 (7), 1285–1294.

Ganser, B.K., Li, S., Klishko, V.Y., Finch, J.T., Sundquist, W.I., 1999. Assembly and analysis of conical models for the HIV-1 core. Science 283 (5398), 80–83.

Ganser, B.K., Cheng, A., Sundquist, W.I., Yeager, M., 2003. Three-dimensional

structure of the M-MuLV CA protein on a lipid monolayer: a general model for retroviral capsid assembly. EMBO J. 22 (12), 2886–2892.
Gitti, R.K., Lee, B.M., Walker, J., Summers, M.F., Yoo, S., Sundquist, W.I., 1996. Structure of the amino-terminal core domain of the HIV-1 capsid protein. Science 273 (5272), 231–235.
Gorelick, R.J., Fu, W., Gagliardi, T.D., Bosche, W.J., Rein, A., Henderson, L.E., Arthur, L.O., 1999. Characterization of the block in replication of nucleocapsid protein zinc finger mutants from Moloney murine leukemia virus. J. Virol. 73 (10), 8185–8195.
Gottlinger, H.G., Sodroski, J.G., Haseltine, W.A., 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. 86 (15), 5781–5785.
Hibbert, C.S., Mirro, J., Rein, A., 2004. mRNA molecules containing murine leukemia virus packaging signals are encapsidated as dimers. J. Virol. 78 (20), 10927–10938.
Jolicoeur, P., Rassart, E., 1980. Effect of Fv-1 gene product on synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. J. Virol. 33 (1), 183–195.
Julias, J.G., McWilliams, M.J., Sarafianos, S.G., Arnold, E., Hughes, S.H., 2002. Mutations in the RNase H domain of HIV-1 reverse transcriptase affect the initiation of DNA synthesis and the specificity of RNase H cleavage in vivo. Proc. Natl. Acad. Sci. U.S.A. 99 (14), 9515–9520.
Kaplan, A.H., Zack, J.A., Knigge, M., Paul, D.A., Kempf, D.J., Norbeck, D.W., Swanstrom, R., 1993. Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. J. Virol. 67 (7), 4050–4055.
Krausslich, H.G., 1992. Specific inhibitor of human immunodeficiency virus proteinase prevents the cytotoxic effects of a single-chain proteinase dimer and restores particle formation. J. Virol. 66 (1), 567–572.
Lee, K., KewalRamani, V.N., 2004. In defense of the cell: TRIM5alpha interception of mammalian retroviruses. Proc. Natl. Acad. Sci. U.S.A. 101 (29), 10496–10497.
Li, S., Hill, C.P., Sundquist, W.I., Finch, J.T., 2000. Image reconstructions of helical assemblies of the HIV-1 CA protein. Nature 407 (6802), 409–413.
Mammano, F., Ohagen, A., Hoglund, S., Gottlinger, H.G., 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. J. Virol. 68 (8), 4927–4936.
Miller, A.D., Miller, D.G., Garcia, J.V., Lynch, C.M., 1993. Use of retroviral vectors for gene transfer and expression. Methods Enzymol. 217, 581–599.
Morgenstern, J.P., Land, H., 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18 (12), 3587–3596.
Mortuza, G.B., Haire, L.F., Stevens, A., Smerdon, S.J., Stoye, J.P., Taylor, I.A., 2004. High-resolution structure of a retroviral capsid hexameric amino-terminal domain. Nature 431 (7007), 481–485.
Oshima, M., Muriaux, D., Mirro, J., Nagashima, K., Dryden, K., Yeager, M., Rein, A., 2004. Effects of blocking individual maturation cleavages in murine leukemia virus gag. J. Virol. 78 (3), 1411–1420.
Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427 (6977), 848–853.
Swanstrom, R., Wills, J.W., 1997. Synthesis, assembly, and processing of viral proteins. In: Coffin, J.M., Hughes, S.H., Varmus, H.E. (Eds.), Retroviruses. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 263–334.
Tang, C., Ndassa, Y., Summers, M.F., 2002. Structure of the N-terminal 283-residue fragment of the immature HIV-1 Gag polyprotein. Nat. Struct. Biol. 9 (7), 537–543.
Tang, S., Murakami, T., Cheng, N., Steven, A.C., Freed, E.O., Levin, J.G., 2003. Human immunodeficiency virus type 1 N-terminal capsid mutants containing cores with abnormally high levels of capsid protein and virtually no reverse transcriptase. J. Virol. 77 (23), 12592–12602.
Towers, G., Bock, M., Martin, S., Takeuchi, Y., Stoye, J.P., Danos, O., 2000. A conserved mechanism of retrovirus restriction in mammals. Proc. Natl. Acad. Sci. U.S.A. 97 (22), 12295–12299.
Trono, D., Feinberg, M.B., Baltimore, D., 1989. HIV-1 Gag mutants can dominantly interfere with the replication of the wild-type virus. Cell 59 (1), 113–120.
von Schwedler, U.K., Stemmler, T.L., Klishko, V.Y., Li, S., Albertine, K.H., Davis, D.R., Sundquist, W.I., 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. EMBO J. 17 (6), 1555–1568.
Yang, W.K., Kiggans, J.O., Yang, D.M., Ou, C.Y., Tennant, R.W., Brown, A., Bassin, R.H., 1980. Synthesis and circularization of N- and B-tropic retroviral DNA Fv-1 permissive and restrictive mouse cells. Proc. Natl. Acad. Sci. U.S.A. 77 (5), 2994–2998.
Yuan, B., Fassati, A., Yueh, A., Goff, S.P., 2002. Characterization of Moloney murine leukemia virus p12 mutants blocked during early events of infection. J. Virol. 76 (21), 10801–10810.

Available online at www.sciencedirect.com
ScienceDirect
Microbes and Infection 8 (2006) 2679–2685
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

NKT cells play a limited role in the neutrophilic inflammatory responses and host defense to pulmonary infection with *Pseudomonas aeruginosa*

Takeshi Kinjo [a], Masashi Nakamatsu [a], Chikara Nakasone [a], Natsuo Yamamoto [a,1], Yuki Kinjo [a,2], Kazuya Miyagi [a], Kaori Uezu [a], Kiwamu Nakamura [a,d], Futoshi Higa [a], Masao Tateyama [a], Kazuyoshi Takeda [b], Toshinori Nakayama [c], Masaru Taniguchi [d], Mitsuo Kaku [e,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 Immunology, Juntendo University School of Medicine, Tokyo, 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] *Department of Infection Control and Laboratory Diagnostics, Internal Medicine, Tohoku University Graduate School of Medicine, Sendai, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8574, Japan*
[f] *CRESCENDO, Japan*
[g] *Infection Control Research Center, Tohoku University Hospital, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8574, Japan*
[h] *Division of Microbiology and Immunology, Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Tohoku University, 2-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8575, Japan*

Received 28 May 2006; accepted 27 July 2006
Available online 22 August 2006

Abstract

CD1d-restricted NKT cells are reported to play a critical role in the host defense to pulmonary infection with *Pseudomonas aeruginosa*. However, the contribution of a major subset expressing a Vα14-Jα18 gene segment remains unclear. In the present study, we re-evaluated the role of NKT cells in the neutrophilic inflammatory responses and host defense to this infection using mice genetically lacking Jα18 or CD1d (Jα18KO or CD1dKO mice). These mice cleared the bacteria in lungs at a comparable level to wild-type (WT) mice. There was no significant difference in the local neutrophilic responses, as shown by neutrophil counts and synthesis of MIP-2 and TNF-α, in either KO mice from those in WT mice. Administration of α-galactosylceramide, a specific activator of Vα14+ NKT cells, failed to promote the bacterial clearance and neutrophilic responses, although the same treatment increased the synthesis of IFN-γ, suggesting the involvement of this cytokine downstream of NKT cells. In agreement against this notion, these responses were not further enhanced by administration of recombinant IFN-γ in the infected Jα18KO mice. Our data indicate that NKT cells play a limited role in the development of neutrophilic inflammatory responses and host defense to pulmonary infection with *P. aeruginosa*.

Keywords: NKT cells; *Pseudomonas aeruginosa*; *Streptococcus pneumoniae*; Host defense; Neutrophils; TNF-α; MIP-2

* Corresponding author. Division of Microbiology and Immunology, Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Tohoku University, 2-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 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.
[2] Present address: La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA, USA.

1286-4579/$ - see front matter © 2006 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2006.07.016

1. Introduction

Pseudomonas aeruginosa, an important causative microorganism of nosocomial pulmonary infection, frequently infects patients with end-stage chronic obstructive lung disorders, cystic fibrosis, and diffuse panbronchiolitis into acute exacerbation caused by overgrowth of this bacterial pathogen [1,2]. It also causes fatal systemic diseases, particularly in immunocompromised patients with chronic debilitating condition, cancer, and burns [3,4]. *P. aeruginosa* is eradicated from infected tissues mainly by a neutrophil-mediated host defense mechanism [5–7]. Upon infection with this bacterium, alveolar macrophages initiate an inflammatory response involving infiltration of a large number of neutrophils from the circulation into the alveolar spaces [8,9].

NKT cells are a lymphocyte subset that acts as a potent regulator of inflammatory responses by rapidly secreting large amounts of cytokines [10,11]. It has been demonstrated that NKT cells played a protective role in the host defense to various infectious pathogens [12]. Mice genetically lacking Vα14+ NKT cells, a major subset of NKT cells (Jα18KO mice) are reported to be highly susceptible to pulmonary infection with *S. pneumoniae*, which correlated with attenuated neutrophil-mediated inflammatory responses [13]. In a study by Nieuwenhuis and co-workers [14], *P. aeruginosa* infection was exacerbated with the reduced accumulation of neutrophils in the lungs of mice with genetic disruption of CD1d gene (CD1dKO mice), which lack the majority of NKT cells [10,11].

Some earlier investigations did not find any evidence for the protective role of Vα14+ NKT cells in the host defense to several infectious pathogens [12]. Indeed, some studies indicated the suppressive role of Vα14+ NKT cells in some infections [12]. In addition, CD1dKO mice lack all CD1d-restricted NKT cells, including some other NKT cell subsets than Vα14+ NKT cells [15]. Therefore, the contribution of Vα14+ NKT cells, a major subset of mouse NKT cells, to the neutrophil-mediated host defense to *P. aeruginosa* remains unclear, including whether it is similar to that reported previously for *S. pneumoniae* infection [13].

Recently, clinical interventions using α-galactosylceramide (α-GalCer), a specific activator of Vα14+ NKT cells [16], have been attempted in patients with malignant diseases [17,18]. Infectious disease caused by *P. aeruginosa* also could be a candidate for such treatment. On these backgrounds, it is important to comprehend the role of this particular subset as well as CD1d-restricted NKT cells in the host defense to *P. aeruginosa* infection. In the present study, we used Jα18KO as well as CD1dKO mice, to re-evaluate the effect of NKT cell deficiency on the clinical course of *P. aeruginosa* infection and neutrophil-mediated inflammatory responses. In contrast to the previous report [14], we failed to find any significant difference in these parameters between NKT cell-deficient and control mice.

2. Materials and methods

2.1. Animals

Vα14+ NKT cell-deficient (Jα18KO) mice were established by targeted deletion of the Jα18 gene [19] and backcrossed eight times with C57BL/6 mice. CD1dKO mice [20] were kindly provided by Luc Van Kaer (Vanderbilt University Graduate School of Medicine, Nashville, TN). These mice were bred in a pathogen-free environment in the Laboratory Animal Center for Biomedical Science, University of the Ryukyus. C57BL/6 mice were purchased from Charles River Japan (Osaka, Japan) and used as a control wild-type (WT) animal. All mice were used at 8–15 weeks of age. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of the University of the Ryukyus.

2.2. Bacteria

P. aeruginosa (PAO-1) were cultured in Trypticase soy broth (Becton Dickinson, Franklin Lake, NJ) at 37 °C, harvested at a mid-log phase, and washed three times in phosphate-buffered saline (PBS). The inoculum was prepared based on its turbidity. A serotype 3, clinical strain of *S. pneumoniae* (URF918) was cultured in Todd-Hewitt broth (Difco, Detroit, MI) at 37 °C in 5% CO_2, and the inoculum was prepared in a similar way. 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 *P. aeruginosa* or *S. pneumoniae* were inoculated at 50 μL per mouse by insertion of an outer sheath of 24G I.V. catheter/needle unit (Becton Dickinson Vascular Access, Sandy, UT) into and parallel to the trachea. In every experiment, a quantification culture was performed to confirm the inoculation dose.

2.3. Enumeration of live bacteria

Mice were sacrificed on Days 1 and 2 (*P. aeruginosa*) or 3 (*S. pneumoniae*) after infection, and the lungs were carefully dissected and excised, and then separately homogenized in 2 or 10 mL of half saline by teasing with a stainless mesh at room temperature. The homogenates, appropriately diluted with half saline, were inoculated at 100 μL on Trypticase soy or 3% sheep blood Muller-Hinton agar plates for *P. aeruginosa* or *S. pneumoniae* and cultured for 18 h, followed by a count of the colonies.

2.4. Treatment with α-GalCer

α-GalCer was kindly provided by Kirin Brewery Co. (Gunma, Japan) and prepared as previously described [21,22]. 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 or

control solution was injected intraperitoneally at 200 μL per mouse on the day of infection.

2.5. Administration of anti-IFN-γ mAb or rIFN-γ

At the time of infection, mice received a single intratracheal administration of neutralizing anti-IFN-γ mAb (R&D, Minneapolis, MN) or control rat IgG (ICN Pharmaceuticals Inc., Aurora, OH) at 25 μg/mouse. In some experiments, mice received a single intratracheal injection of mouse recombinant (r)IFN-γ (PeproTech Inc., Rocky Hill, NJ) at 50 ng/mouse or PBS as a control.

2.6. Preparation of BAL fluids

Bronchoalveolar lavage (BAL) fluid samples were collected as described below. Briefly, after bleeding under anesthesia with isoflurane, the chest was opened and the trachea was cannulated with an outer sheath of 24G I.V. catheter/needle unit (Becton Dickinson Vascular Access), followed by lavage of the lung three times with 1 mL of chilled PBS. About 1×10^5 cells were centrifuged onto a glass slide at 800 rpm for 3 min using Auto Smear CF-12D (Sakura Co., Tokyo), and stained using the May-Giemsa technique. To analyze the leukocyte fraction, at least 500 cells were examined by photomicroscopic examination. The number of neutrophils was calculated by multiplying the leukocyte number in BAL fluids by their proportion.

2.7. Measurement of cytokine concentrations

The concentrations of MIP-2, TNF-α and IFN-γ in the lung homogenates, prepared by teasing with the stainless mesh without collagenase and DNase treatment, and culture supernatants were measured by the respective enzyme-linked immunosorbent assay (ELISA) kit (R&D). The detection limits of assays for MIP-2, TNF-α and IFN-γ were 1.5, 5.1 and 2 pg/mL, respectively.

2.8. 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 the ANOVA test with a post-hoc analysis (Fisher PLSD test). A p value less than 0.05 was considered significant.

3. Results

3.1. Host defense to P. aeruginosa infection in NKT cell-deficient mice

To determine the role of Vα14+ NKT cells in the host defense to *P. aeruginosa* infection, we established a mouse model of acute pulmonary infection. In this model, bacteria were gradually eradicated with time from the lungs. Initially, we compared the clinical course of *P. aeruginosa* infection by counting the number of live colonies in the lungs of WT and Jα18KO mice. As shown in Fig. 1A, bacterial counts were slightly lower in Jα18KO mice than WT mice on Days 1 and 2 at a high-dose infection (1.5×10^6 and 1.2×10^6 CFU/mouse, respectively), and there was no apparent difference in these groups on Day 2 at mid- and low-dose infection (4.6×10^5 and 1.8×10^5 CFU/mouse, respectively). Similar results were obtained when CD1dKO mice were infected (Fig. 1B).

3.2. Neutrophilic inflammatory responses to P. aeruginosa infection in NKT cell-deficient mice

Neutrophils play a critical role in the host protection against *P. aeruginosa* infection [5–7]. MIP-2 and TNF-α are important cytokines in their recruitment to the inflammatory sites [23–26]. Therefore, we next compared the accumulation of neutrophils and synthesis of these cytokines in the lungs between WT and Jα18KO or CD1dKO mice. In preliminary experiments, the number of neutrophils in BAL fluids reached a peak level at 6 h after infection (data not shown), which made us to select this time point in the following experiments. As shown in Fig. 1C, such number was not significantly different among these mice. Similar results were observed in the synthesis of MIP-2 and TNF-α in the lungs. There was no significant difference in the production of these cytokines, except for TNF-α which showed a small increase in CD1dKO mice, at 3 h after infection with *P. aeruginosa*, a time point earlier than the maximal accumulation of neutrophils (Fig. 1D).

3.3. Effect of α-GalCer treatment on the host defense to P. aeruginosa infection

To further address the role of Vα14+ NKT cells in the host defense to *P. aeruginosa* infection, we tested the effect of α-GalCer treatment on the clinical course and neutrophil-mediated host responses in WT mice by comparing with those in *S. pneumoniae* infection. As shown in Fig. 2A, this treatment significantly reduced the lung burden in *S. pneumoniae* infection, but not in *P. aeruginosa*. The number of neutrophils accumulated in BAL fluids was drastically reduced by α-GalCer treatment in *P. aeruginosa* infection, whereas by a striking contrast, this number was significantly increased in case of *S. pneumoniae* (Fig. 2B). The synthesis of MIP-2 and TNF-α in the lungs was not affected by the same treatment in *P. aeruginosa*, which was not parallel to the reduction in neutrophil counts, whereas such synthesis was significantly increased in *S. pneumoniae* infection. Interestingly, IFN-γ production was induced by α-GalCer treatment at a comparable level in both infections (Fig. 2C).

3.4. Role of IFN-γ in the host response to P. aeruginosa infection

The results shown in Fig. 2C raised a possibility that the contribution of IFN-γ to neutrophil-mediated responses was

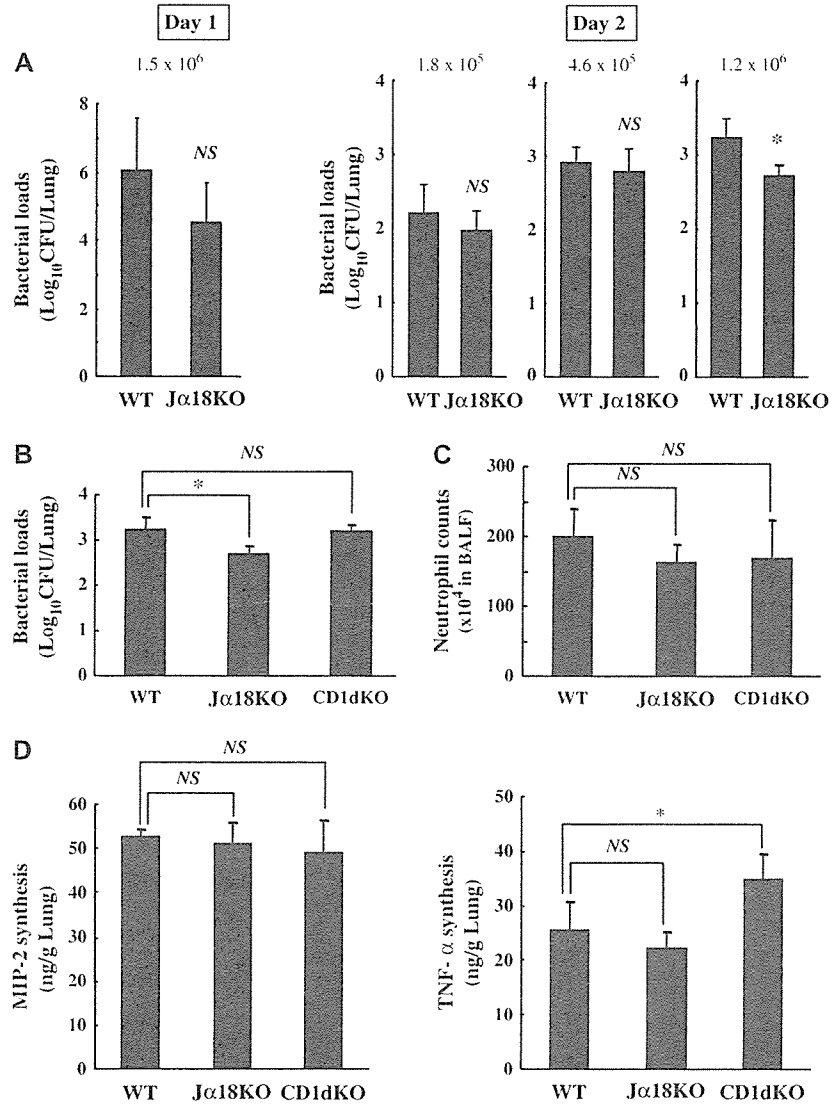

Fig. 1. Host protective response in Jα18KO and CD1dKO mice infected with *P. aeruginosa*. WT, Jα18KO or CD1dKO mice were infected intratracheally with *P. aeruginosa* at indicated doses per mouse (A); at 1.2×10^6 (B); at 1.1×10^6 (C); or at 1.5×10^6 per mouse (D). The number of live colonies in lungs was counted on Days 1 and 2 (A); or Day 2 post-infection (B). The number of neutrophils in BAL fluids was counted at 6 h post-infection (C); and the concentration of MIP-2 and TNF-α in lung homogenates was measured at 3 h post-infection (D). Each column represents the mean ± SD of six mice. Experiments were repeated three times with similar results. *NS*, not significant; $^*p < 0.05$, compared to WT mice.

different in these two bacterial infections. To address this possibility, we examined the effect of neutralizing anti-IFN-γ mAb on the synthesis of MIP-2 and TNF-α caused by infection in WT mice. As shown in Fig. 3, such synthesis was significantly reduced by anti-IFN-γ mAb treatment in *S. pneumoniae* infection, whereas there was no significant influence in case of *P. aeruginosa*. Because NKT cells have been known to promptly secrete a large amount of IFN-γ [10,11], we further tested whether administration of rIFN-γ affected the host defense and synthesis of MIP-2 and TNF-α in Jα18KO mice. In *S. pneumoniae* infection, administration of rIFN-γ significantly promoted the clearance of bacteria and synthesis of both cytokines to a comparable level to those in WT mice, whereas the same treatment did not affect theses values, but rather increased the number of live colonies, in *P. aeruginosa* infection (Fig. 4A,B). These results suggest that the role of IFN-γ in the host defense to *P. aeruginosa* infection is limited by contrast to *S. pneumoniae*, in which this cytokine may play some role downstream of NKT cells.

4. Discussion

In the present study, we re-evaluated the role of NKT cells in the neutrophil-mediated host defense to *P. aeruginosa* infection using Jα18KO as well as CD1dKO mice. The clearance of bacteria, accumulation of neutrophils and synthesis of MIP-2 and TNF-α in lungs were not significantly different between WT and Jα18KO or CD1dKO mice. These results were a sharp contrast to those in *S. pneumoniae* infection, in which all these parameters were significantly attenuated in Jα18KO mice compared to WT mice [13]. These observations suggested that NKT cells play a limited role in the neutrophil-mediated

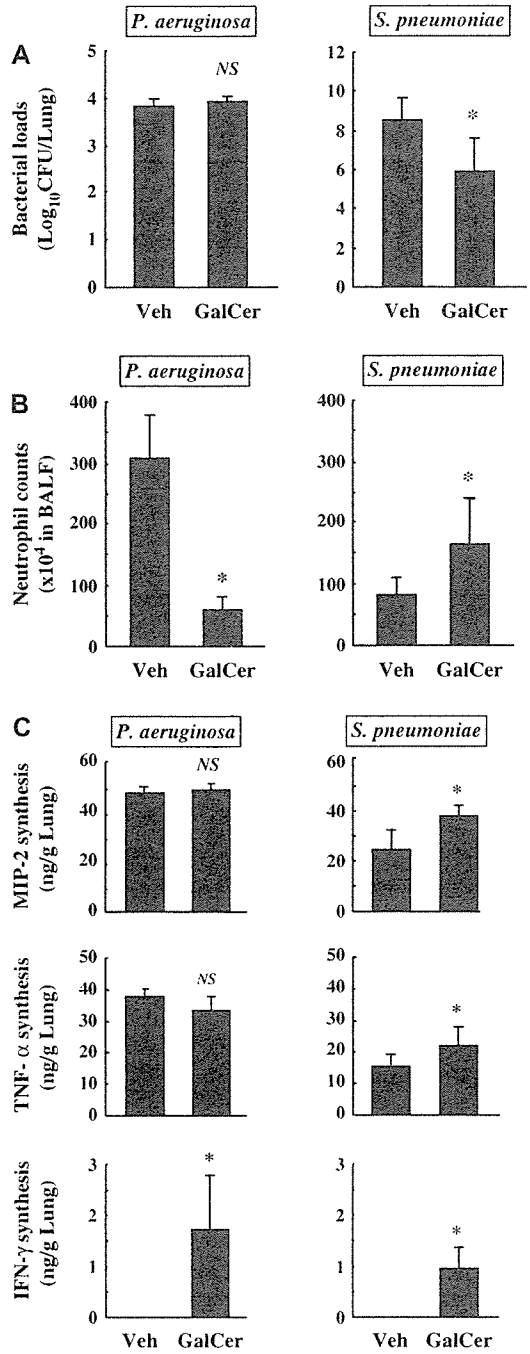

Fig. 2. Effect of α-GalCer on the infection with *P. aeruginosa* and *S. pneumoniae*. WT mice were infected intratracheally with *P. aeruginosa* or *S. pneumoniae* at 1.3 to 2.7 × 10^6 or 1.5 to 3.0 × 10^6 per mouse, respectively. These mice received intraperitoneal injections of α-GalCer (2 μg/mouse) or vehicle control on the day of infection. (A) The number of live colonies was counted on Day 2 or 3 post-infection, respectively. (B) The number of neutrophils in BAL fluids was counted at 6 h post-infection. (C) The concentration of MIP-2, TNF-α and IFN-γ in lung homogenates was measured at 3 h post-infection. Each column represents the mean ± SD of six mice. Experiments were repeated three times with similar results. Veh, vehicle; GalCer, α-galactosylceramide. *NS*, not significant; *$p < 0.05$, compared to vehicle control.

inflammatory responses and host resistance to *P. aeruginosa* infection.

In an earlier study by Nieuwenhuis and co-workers [14], CD1dKO mice were highly susceptible to pulmonary infection

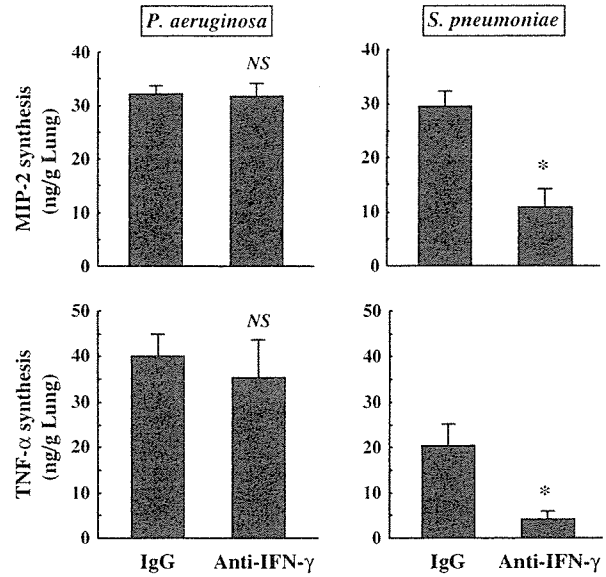

Fig. 3. Effect of anti-IFN-γ mAb treatment on MIP-2 and TNF-α synthesis in lungs. WT mice were infected intratracheally with *P. aeruginosa* or *S. pneumoniae* at 1.3 × 10^6 or 1.7 × 10^6 per mouse, respectively. These mice received an intratracheal injection of anti-IFN-γ mAb or control IgG. The concentration of MIP-2 and TNF-α in lung homogenates was measured at 3 h post-infection. Each column represents the mean ± SD of four mice. Experiments were repeated twice with similar results. *NS*, not significant; *$p < 0.05$, compared to control IgG.

with *P. aeruginosa*, suggesting a critical role for CD1d-restricted NKT cells in the host defense to this bacterial pathogen. These data are not consistent with our present observations. Although the precise reason remains unclear, some distinct experimental conditions including bacterial strains (D4 *vs.* PAO-1) and routes of infection (intranasal *vs.* intratracheal) might partially account for this discrepancy. These investigators also did not use Jα18KO mice to define the role of Vα14+ NKT cells in this infection. Different roles of diverse CD1d-restricted NKT cells and Vα14+ NKT cells have been reported in a different situation [12]. *L. monocytogenes* infection is alleviated in mice receiving anti-CD1d mAb, while Jα18KO mice are highly susceptible to the same infectious pathogen. In *Trypanosoma cruzi* infection, CD1d-restricted NKT cells play a proinflammatory role, while Vα14+ NKT cells show an anti-inflammatory effect [12,27]. These data suggest that these subsets of NKT cells may differentially contribute to the inflammatory responses caused by infection. Similar results were reported in CD1dKO and Jα18KO mice after infection with *M. tuberculosis*, in which both mice were equally resistant to this infection [12]. Thus, investigations using CD1dKO and Jα18KO mice may not necessarily produce a similar conclusion with respect to the role of NKT cells in the host defense to infection.

In addition, Nieuwenhuis and co-workers reported that α-GalCer treatment led to enhanced clearance of *P. aeruginosa* in lungs, which was associated with production of IFN-γ and increased phagocytosis of the bacteria by macrophages, while number of neutrophils accumulated in the lungs was strikingly reduced by the same treatment [14]. These authors

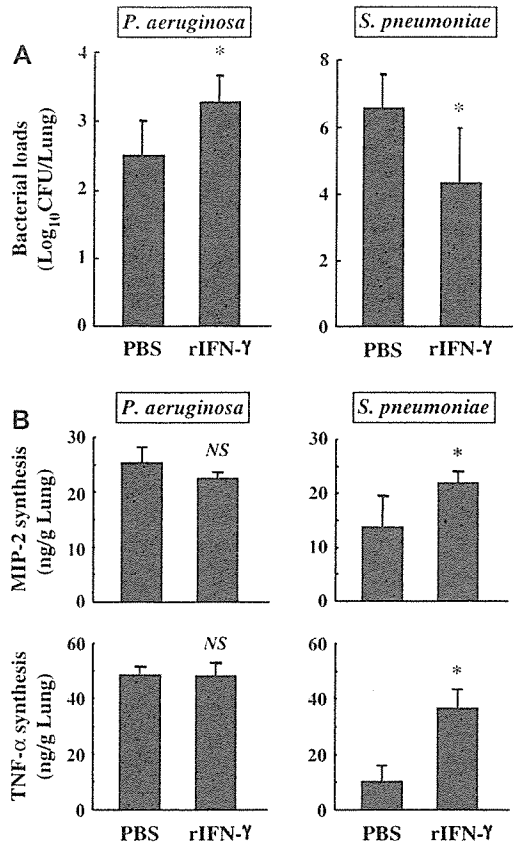

Fig. 4. Effect of rIFN-γ administration on the bacterial clearance and cytokine synthesis in lungs. Jα18KO mice were infected intratracheally with *P. aeruginosa* or *S. pneumoniae* at 7.1×10^6 or 3.0×10^5 (A); and 7.1×10^6 or 6.1×10^6 per mouse (B), respectively. These mice received an intratracheal injection of rIFN-γ or PBS. The number of live colonies (A); and concentration of MIP-2 and TNF-α in lung homogenates (B) were measured on day 2 or 3 and at 3 h post-infection, respectively. Each column represents the mean ± SD of six to seven mice (A); and four mice (B). Experiments were repeated twice with similar results. *NS*, not significant; $^*p < 0.05$, compared to PBS.

speculate that such promoted host defense is ascribed to IFN-γ-induced clearance of this bacterium by macrophages. Our study showed similar results in the reduced accumulation of neutrophils in the infected lungs after treatment with α-GalCer, the mechanism of which remains to be understood. By contrast, clearance of *P. aeruginosa* and synthesis of MIP-2 and TNF-α associated with neutrophil-mediated inflammatory responses were not affected by α-GalCer treatment. These data are not inconsistent with the fact that we failed to detect any influence of Vα14+ NKT cell-deficiency on the clinical course and neutrophil-mediated inflammatory responses after *P. aeruginosa* infection. These were in a striking contrast to the data observed in *S. pneumoniae* infection, in which α-GalCer treatment significantly promoted the clearance of this bacterium, accumulation of neutrophils, and synthesis of MIP-2 and TNF-α in the infected tissues, which correlated well with the increased susceptibility of Vα14+ NKT cell-deficient mice to this infection. In contrast to our data in *P. aeruginosa* infection, previous investigations showed that these mice are not prone to *M. tuberculosis* infection [12], but α-GalCer treatment promotes the host resistance to this infection [28]. To date, the precise mechanism to account for the different effects of α-GalCer treatment remains to be fully understood and will require further investigations.

Activation of Vα14+ NKT cells by α-GalCer resulted in the synthesis of IFN-γ in lungs that was equivalent to that observed after infection with both bacterial pathogens; this effect was not observed in MIP-2 and TNF-α production in *P. aeruginosa* infection in contrast to that caused by *S. pneumoniae*. These observations raised a possibility that the differential role of Vα14+ NKT cells in the host defense to these infections may be due to the distinct effect of IFN-γ derived from this particular lymphocyte subset on the synthesis of MIP-2 and TNF-α. To address this possibility, we tested the effect of neutralizing anti-IFN-γ mAb or rIFN-γ on the host protective responses to infection. Our findings showed that manipulations suppressing or activating this cytokine did not make any impact on these responses in *P. aeruginosa* infection, which was in a striking contrast to *S. pneumoniae* infection, suggesting a limited role of this cytokine in the inflammatory responses caused by *P. aeruginosa* infection. In agreement with this notion, a previous study by Schultz and co-workers [29] demonstrated that IFN-γ was not critical to the host resistance to infection with *P. aeruginosa*.

In conclusion, the present study demonstrated that neither diverse CD1d-restricted nor Vα14+ NKT cells played a critical role in the neutrophil-mediated host defense to *P. aeruginosa* infection, which was in contrast to *S. pneumoniae* infection. Although the reason for this difference remains unclear, the present findings may further enhance our understanding of the role of NKT cells in the host defense to bacterial infection.

Acknowledgements

The authors thank Dr. Luc Van Kaer (Vanderbilt University Graduate School of Medicine, Nashville, TN) for a kind gift of CD1dKO mice. 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".

References

[1] G.B. Pier, Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interactions, J. Infect. Dis. 151 (1985) 575–580.
[2] N. Keicho, S. Kudoh, Diffuse panbronchiolitis: role of macrolides in therapy, Am. J. Respir. Med. 1 (2002) 119–131.
[3] I.M. Gould, R. Wise, *Pseudomonas aeruginosa*: clinical manifestations and management, Lancet 2 (8466) (1985) 1224–1227.
[4] G.P. Bodey, R. Bolivar, V. Fainstein, L. Jadeja, Infections caused by *Pseudomonas aeruginosa*, Rev. Infect. Dis. 5 (1983) 279–313.
[5] 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. 479–492.
[6] G. Doring, Host response to *Pseudomonas aeruginosa*, Acta Paediatr. Scand. Suppl. 363 (1989) 37–39.

[7] A. Kadioglu, P.W. Andrew, The innate immune response to pneumococcal lung infection: the untold story, Trends Immunol. 25 (2004) 143–149.
[8] G.B. Toews, E.J. Hansen, R.M. Strieter, Pulmonary host defenses and oropharyngeal pathogens, Am. J. Med. 88 (1990) 20S–24S.
[9] J.P. Mizgerd, Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs, Semin. Immunol. 14 (2002) 123–132.
[10] 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.
[11] M. Kronenberg, Toward an understanding of NKT cell biology: progress and paradoxes, Annu. Rev. Immunol. 23 (2005) 877–900.
[12] Y. Kinjo, M. Kronenberg, Valpha14i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes, J. Clin. Immunol. 25 (2005) 522–533.
[13] 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.
[14] E.E. Nieuwenhuis, T. Matsumoto, M. Exley, R.A. Schleipman, J. Glickman, D.T. Bailey, N. Corazza, S.P. Colgan, A.B. Onderdonk, R.S. Blumberg, CD1d-dependent macrophage-mediated clearance of *Pseudomonas aeruginosa* from lung, Nat. Med. 8 (2002) 588–593.
[15] D.I. Godfrey, H.R. MacDonald, M. Kronenberg, M.J. Smyth, L.V. Kaer, NKT cells: what's in a name? Nat. Rev. Immunol. 4 (2004) 231–237.
[16] T. Kawano, J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, M. Taniguchi, CD1d-restricted and TCR-mediated activation of Valpha14 NKT cells by glycosylceramides, Science 278 (1997) 1626–1629.
[17] K. Seino, S. Fujii, M. Harada, S. Motohashi, T. Nakayama, T. Fujisawa, M. Taniguchi, Valpha14 NKT cell-mediated anti-tumor responses and their clinical application, Springer Semin. Immun. 27 (2005) 65–74.
[18] M. Terabe, J.A. Berzofsky, Immunoregulatory T cells in tumor immunity, Curr. Opin. Immunol. 16 (2004) 157–162.
[19] 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 non-specific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5690–5693.
[20] S.K. Mendiratta, W.D. Martin, S. Hong, A. Boesteanu, S. Joyce, L. Van Kaer, CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4, Immunity 6 (1997) 469–477.
[21] 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.
[22] E. Kobayashi, M. Motoki, T. Uchida, H. Fukushima, Y. Koezuka, KRN7000, a novel immunomodulator, and its antitumor activities, Oncol. Res. 7 (1995) 529–534.
[23] M.R. Jones, B.T. Simms, M.M. Lupa, M.S. Kogan, J.P. Mizgerd, Lung NF-kappaB activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia, J. Immunol. 175 (2005) 7530–7535.
[24] A.W. Rijneveld, G.P. van den Dobbelsteen, S. Florquin, T.J. Standiford, P. Speelman, L. van Alphen, T. van der Poll, Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice, J. Infect. Dis. 185 (2002) 123–126.
[25] J. Lee, L.D. Sorbo, A.A. Khine, J. de Azavedo, D.E. Low, D. Bell, S. Uhlig, A.S. Slutsky, H. Zhang, Modulation of bacterial growth by tumor necrosis factor-alpha in vitro and in vivo, Am. J. Respir. Crit. Care Med. 168 (2003) 1462–1470.
[26] W.C. Tsai, R.M. Strieter, B. Mehrad, 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.
[27] M.S. Duthie, M. Kahn, M. White, R.P. Kapur, S.J. Kahn, Critical proinflammatory and anti-inflammatory functions of different subsets of CD1d-restricted natural killer T cells during *Trypanosoma cruzi* infection, Infect. Immun. 73 (2005) 181–192.
[28] A. Chackerian, J. Alt, V. Perera, S.M. Behar, Activation of NKT cells protects mice from tuberculosis, Infect. Immun. 70 (2002) 6302–6309.
[29] M.J. Schultz, A.W. Rijneveld, P. Speelman, S.J. van Deventer, T. van der Poll, Endogenous interferon-gamma impairs bacterial clearance from lungs during *Pseudomonas aeruginosa* pneumonia, Eur. Cytokine. Netw. 12 (2001) 39–44.