BM-DCs more rapidly than either agent alone, and synergistically enhances cytokine production.

Requirement of TLR2 Ligand for Enhanced Cytokine Production by BM-DCs in Response to Poly(I:C) and Zymosan

Lastly, the mechanism was examined by which zymosan enhanced the production of cytokines in combination with poly(I:C). Zymosan is composed of 55% β-glucan, which is a ligand of Dectin-1, and an unknown ligand of TLR2 [Di Carlo and Fiore, 1958; Gantner et al., 2003; Slack et al., 2007]. To determine which of these components of zymosan contributed to the synergistic effect on cytokine production in BM-DCs, cells were treated with Pam_3CSK_4 (0.1 or 1 μg), which is a TLR2-specific ligand, or β-glucan (0.1, 1, or 10 μg), either alone or in the presence of 5 μg poly(I:C). As shown in Figure 5, TNF-α production in the presence of 1 μg of Pam_3CSK_4 and 5 μg of poly(I:C) was 100- and 3-fold higher than in the presence of Pam_3CSK_4 and poly(I:C) alone, respectively. Similarly, the production of IL-6, IL-12p70 and IL-10 in response to 1 μg of Pam_3CSK_4 was increased significantly by co-treatment with 5 μg of poly(I:C). By comparison, when BM-DCs were cultured in the presence of β-glucan, there were no significant differences in cytokine production in the presence or absence of poly(I:C). Furthermore, there were no significant differences in the expression of CD86 and CD40 in cells cultured with β-glucan in the presence or absence of poly(I:C) (data not shown). These results suggest that the ability of zymosan to enhance the production of cytokines in BM-DCs in the presence of poly(I:C) is due to the TLR2 ligand component of zymosan.

DISCUSSION

The development of an effective mucosal influenza vaccine is very important if nasal vaccines are to be deployed instead of parenteral vaccines. The development of an effective inactivated mucosal vaccine requires that a suitable mucosal adjuvant be used with the vaccine. Previously, it was shown that the synthetic double-stranded RNA poly(I:C) is an effective adjuvant when administered with an inactivated nasal influenza vaccine [Ichinohe et al., 2005]. In the current study, in an attempt to enhance further the effectiveness of the nasal influenza vaccine, the effect of zymosan was investigated on the adjuvant activity of poly(I:C) in mice immunized intranasally with an inactivated influenza vaccine (1 μg). Zymosan (10 μg) plus poly(I:C) (1–5 μg) increased synergistically the levels of nasal IgA and serum IgG antibodies in immunized mice (Fig. 1). This synergistic increase in antibody production resulted in an enhanced ability of the immunized mice to mount a protective response to influenza virus challenge and protected mice from viral pneumonia (Fig. 2), with a corresponding increase in survival rate without body weight loss (Fig. 3). These results suggest that zymosan is an effective adjuvant for enhancing the effectiveness of the poly(I:C)-combined nasal influenza vaccine.

The mechanism by which zymosan enhanced the adjuvant activity of poly(I:C) was investigated using an in vitro BM-DC culture system. Zymosan is a cell wall extract from *S. cervisiae* that contains β-glucan (55%), mannan, protein, lipid, chitin [Di Carlo and Fiore, 1958] and an unknown TLR2 ligand [Gantner et al., 2003; Slack et al., 2007]. Zymosan has been used for over 50 years as a model microorganism to investigate phagocytosis and the inflammatory response both in vivo and in vitro [Di Carlo and Fiore, 1958]. β-glucan, one of components of zymosan, is recognized by Dectin-1, a C-type lectin that mediates phagocytosis of microbial agents by DCs or macrophages [Herre et al., 2004; Brown, 2006; Robinson et al., 2006]. In preliminary experiments, it was confirmed that zymosan facilitates the uptake of FITC-labeled HA vaccines prepared from A/Yamagata virus (H1N1) by BM-DCs (unpublished

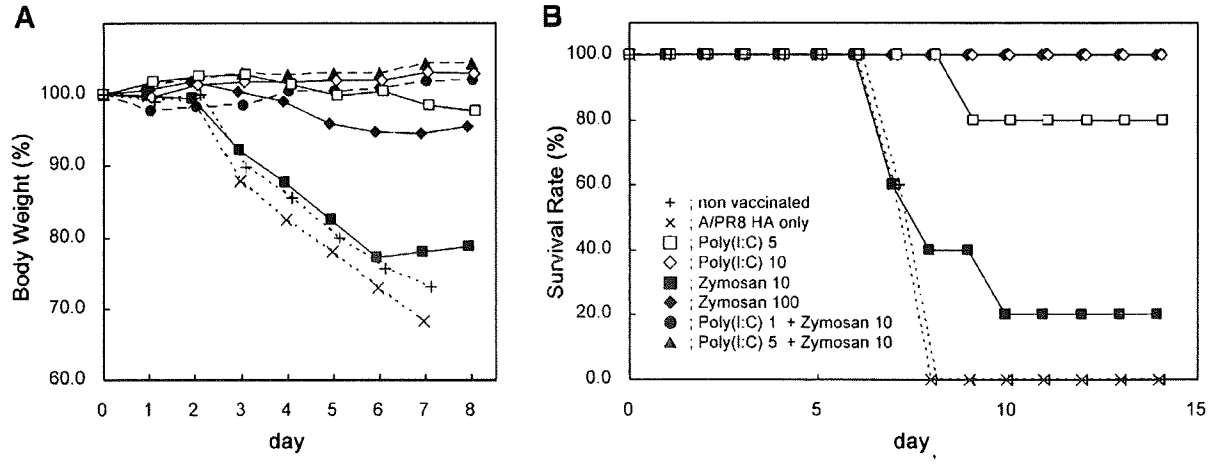

Fig. 3. Survival and body weight of mice immunized and infected with influenza virus. Mice were immunized as described for Figure 1 and then infected with a large volume of A/PR8 virus. Changes in body weight (**A**) and survival rate (**B**) were monitored for 8 and 14 days after infection, respectively. Body weight is represented as relative to the initial mean body weight of five mice.

data). Thus, β-glucan appears to be involved in the uptake of HA vaccine by BM-DCs in the presence of zymosan.

When microbial pathogens or structures, such as zymosan, are phagocytosed, surface TLRs, including TLR4 and TLR2, are recruited to the phagosome [Blander and Medzhitov, 2006]. TLR-mediated intracellular signaling pathways are divided into two main categories, MyD88-dependent and TRIF-dependent signaling pathways, depending on the type of adaptor molecule that is engaged [Takeda and Akira, 2005]. Simultaneous or sequential stimulation of MyD88-dependent and TRIF-dependent signaling pathways by their respective ligands induces a synergistic increase in the production of TNF-α, IL-6, and IFN-β; although tolerance is induced by agonists that act through the same pathway [Bagchi et al., 2007]. A similar result was noted in the current study, in that poly(I:C), a ligand of TLR3/TRIF, when combined with Pam_3CSK_4 which is a TLR2 ligand and activator of TLR2/MyD88 signaling, synergistically enhanced the production of various cytokines (Fig. 5). On the other hand, poly(I:C) in combination with β-glucan, also a constituent of zymosan and an activator of Dectin-1/Syk signaling, had no such effect on cytokine production (Fig. 5). These results suggest that simultaneous stimulation of MyD88-dependent and TRIF-dependent signaling pathways by their respective ligands induces a synergistic increase in cytokine production.

The mechanism by which an inactivated influenza vaccine in the presence of poly(I:C) and zymosan is recognized by DCs and induces an elevated antibody response likely involves many steps. The vaccine is taken up by DCs by phagocytosis and provides a potential source of peptides that can bind to major histocompatibility complex (MHC) class II molecules on the surface of the DC [Blander and Medzhitov, 2006]. Antigenic recognition of the complex of peptide/MHC class II on the surface of DCs by $CD4^+$ T cells induces antibody production. The β-glucan component of zymosan, which is recognized by Dectin-1, might facilitate the phagocytosis of zymosan [Robinson et al., 2006] together with the vaccine, whereas poly(I:C) can be endocytosed by DCs and bind to intracellular TLR3, perhaps within the endosome [Iwasaki and Medzhitov, 2004; Kawai and Akira, 2006]. Activation of two different signaling pathways, TLR3/TRIF by poly(I:C) and TLR2/MyD88 by zymosan, results in the synergistic increase in cytokine production by DCs. Alternatively, the synergistic effect of the two adjuvants might be accomplished within the same endosomal compartment, as poly(I:C) could be phagocytosed simultaneously with zymosan, and phagosomes could fuse with endosomes containing TLR3 during the process of phagosome maturation. In the current study, the levels of cytokines involved in the activation of the innate immune response (IFN-β, IL-6, and TNF-α) as well as the acquired immune response (IL-6, IL-10, and IL-12) were increased synergistically in the presence of poly(I:C) and zymosan (Figs. 4 and 5). These results suggest a possible mechanism by which zymosan enhances the activity of poly(I:C) as an adjuvant of the influenza HA vaccine: (1) stimulation

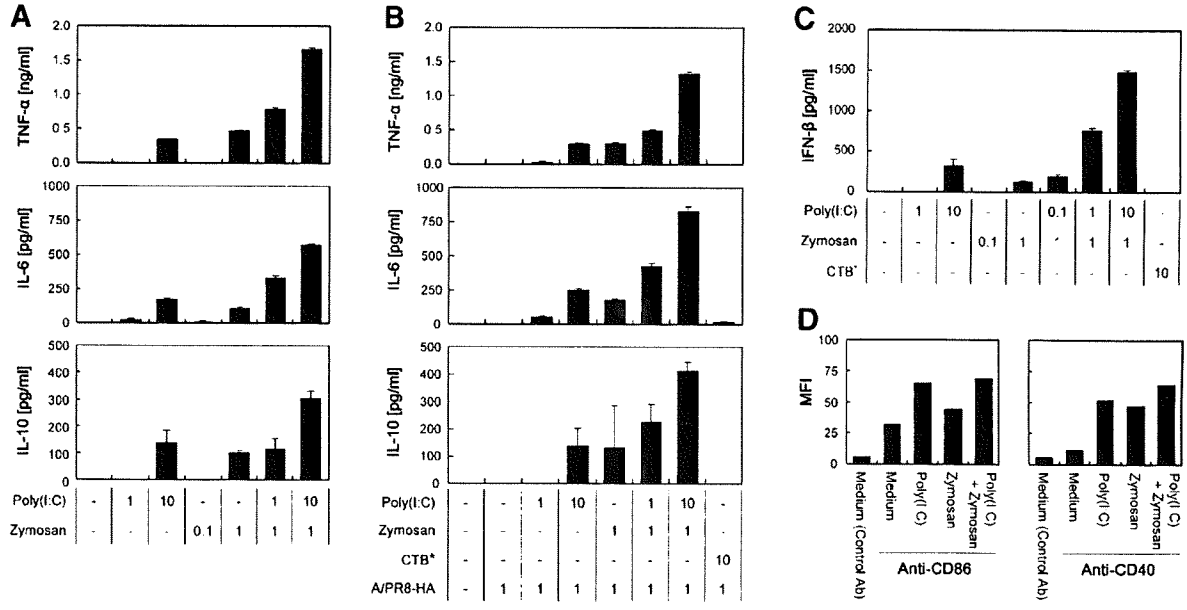

Fig. 4. Cytokine production by BM-DCs and maturation of BM-DCs in the presence of poly(I:C) and/or zymosan. BM-DCs (5×10^5 cells/well) were cultured for 24 hr in the presence of poly(I:C) (0.1, 1, or 10 μg) or zymosan (0.1 or 1 μg) alone, or with both, in the absence (A and C) or presence (B) of an A/PR8 HA vaccine (1 μg). The production of TNF-α, IL-12p70, and IL-10 (A and B) was measured by sandwich ELISA. IFN-β (C) was also quantified by ELISA. ELISAs were performed in triplicate for each sample. Each data is representative of two independent experiments, and represent the means ± standard deviation (SD). (D) BM-DCs (5×10^5 cells/well) were cultured with poly(I:C) (10 μg) or zymosan (1 μg) alone, or with both, and then the expression of the co-stimulatory molecules CD86 and CD40 was analyzed flow cytometry. Data represents the mean fluorescence intensity (MFI) for each molecule within the population of CD11c-positive BM-DCs.

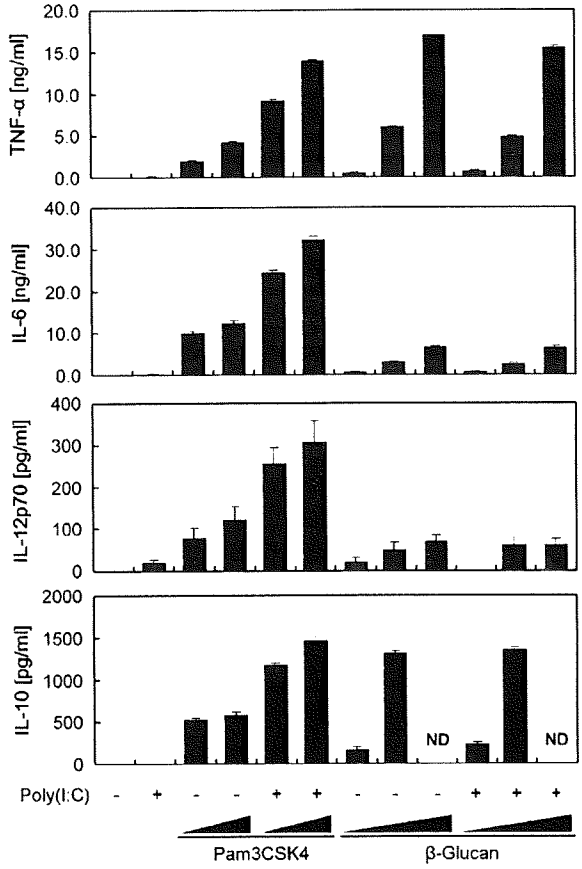

Fig. 5. Cytokine production by BM-DCs in the presence of poly(I:C) and either Pam3CSK4 or β-glucan. BM-DCs (5×10^5 cells/well) were cultured for 24 hr in the presence of poly(I:C) (5 μg), Pam_3CSK_4 (0.1 or 1 μg), or β-glucan (0.1, 1, or 10 μg) alone, or the indicated combinations of the three molecules. The production of TNF-α, IL-6, IL-12p70, and IL-10 in culture supernatants was determined by ELISA. Data represent the means ± SD of two or three independent experiments. ND; not done.

of Dectin-1-dependent phagocytosis of the HA vaccine by DCs in the presence zymosan; (2) synergistic enhancement of cytokine production by the stimulation of both TLR3/TRIF and TLR2/MyD88 signaling pathways in the presence of poly(I:C) and an unknown TLR2 ligand; and (3) the activation of $CD4^+$ T cells, which recognize influenza peptide/MHC class II complexes on the surface of DCs, and facilite the proliferation and differentiation of T and B cells by various cytokines.

ACKNOWLEDGMENTS

We thank Mr. S. Nagasaka (Tokyo University of Science, Chiba, Japan) for technical suggestions and helpful discussions.

REFERENCES

Asahi Y, Yoshikawa T, Watanabe I, Iwasaki T, Hasegawa H, Sato Y, Shimada S, Nanno M, Matsuoka Y, Ohwaki M, Iwakura Y, Suzuki Y, Aizawa C, Sata T, Kurata T, Tamura S. 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J Immunol 168:2930–2938.

Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, Hellman J. 2007. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. J Immunol 178:1164–1171.

Blander JM, Medzhitov R. 2006. On regulation of phagosome maturation and antigen presentation. Nat Immunol 7:1029–1035.

Brown GD. 2006. Dectin-1: A signalling non-TLR pattern-recognition receptor. Nat Rev Immunol 6:33–43.

Couch RB, Kasel JA. 1983. Immunity to influenza in man. Annu Rev Microbiol 37:529–549.

Davenport FM, Hennessy AV, Brandon FM, Webster RG, Barrett CD, Jr., Lease GO. 1964. Comparisons of serologic and febrile responses in humans to vaccination with influenza a viruses or their hemagglutinins. J Lab Clin Med 63:5–13.

Di Carlo FJ, Fiore JV. 1958. On the composition of zymosan. Science 127:756–757.

Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med 197:1107–1117.

Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, Forster I, Ruland J. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature 442:651–656.

Hasegawa H, Ichinohe T, Strong P, Watanabe I, Ito S, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T. 2005. Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. J Med Virol 75:130–136.

Herre J, Marshall AS, Caron E, Edwards AD, Williams DL, Schweighoffer E, Tybulewicz V, Reis e Sousa C, Gordon S, Brown GD. 2004. Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. Blood 104:4038–4045.

Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T, Hasegawa H. 2005. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 79:2910–2919.

Ichinohe T, Watanabe I, Tao E, Ito S, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Moriyama M, Chiba J, Komase K, Suzuki Y, Kurata T, Sata T, Hasegawa H. 2006. Protection against influenza virus infection by intranasal vaccine with surf clam microparticles (SMP) as an adjuvant. J Med Virol 78:954–963.

Iwasaki A, Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol 5:987–995.

Kamijuku H, Nagata Y, Jiang X, Ichinohe T, Tashiro T, Mori K, Taniguchi M, Hase K, Ohno H, Shimaoka T, Yonehara S, Odagiri T, Tashiro M, Sata T, Hasegawa H, Seino KI. 2008. Mechanism of NKT cell activation by intranasal coadministration of alpha-galactosylceramide, which can induce cross-protection against influenza viruses. Mucosal Immunol 1:208–218.

Kawai T, Akira S. 2006. Innate immune recognition of viral infection. Nat Immunol 7:131–137.

Lamb RA, Krug RM. 2001. Orthomyxoviridae: The viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin A M, Roizman B, Straus SE, editors. Fields virology. 4th edition. Philadelphia: Lippincott Williams & Wilkins. pp. 1487–1531.

Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur J Immunol 14:350–356.

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223:77–92.

Murphy BR, Clements ML. 1989. The systemic and mucosal immune response of humans to influenza A virus. Curr Top Microbiol Immunol 146:107–116.

O'Neill LA. 2008. When signaling pathways collide: Positive and negative regulation of toll-like receptor signal transduction. Immunity 29:12–20.

Robinson MJ, Sancho D, Slack EC, LeibundGut-Landmann S, Reis e Sousa C. 2006. Myeloid C-type lectins in innate immunity. Nat Immunol 7:1258–1265.

Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL, Brown GD, Reis e Sousa C. 2005. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity 22:507–517.

Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, Kinjo T, Nakamura K, Kawakami K, Iwakura Y. 2007. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat Immunol 8:39–46.

Sever JL. 1962. Application of a microtechnique to viral serological investigations. J Immunol 88:320–329.

Slack EC, Robinson MJ, Hernanz-Falcon P, Brown GD, Williams DL, Schweighoffer E, Tybulewicz VL, Reis e Sousa C. 2007. Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan. Eur J Immunol 37:1600–1612.

Takeda K, Akira S. 2005. Toll-like receptors in innate immunity. Int Immunol 17:1–14.

Tamura S, Ito Y, Asanuma H, Hirabayashi Y, Suzuki Y, Nagamine T, Aizawa C, Kurata T. 1992a. Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. J Immunol 149:981–988.

Tamura SI, Asanuma H, Ito Y, Hirabayashi Y, Suzuki Y, Nagamine T, Aizawa C, Kurata T, Oya A. 1992b. Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. Eur J Immunol 22:477–481.

Tamura S, Yamanaka A, Shimohara M, Tomita T, Komase K, Tsuda Y, Suzuki Y, Nagamine T, Kawahara K, Danbara H, Aizawa C, Oya A, Kurata T. 1994. Synergistic action of cholera toxin B subunit (and Escherichia coli heat-labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. Vaccine 12:419–426.

Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, Haynes K, Steele C, Botto M, Gordon S, Brown GD. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol 8:31–38.

Tobita K, Sugiura A, Enomote C, Furuyama M. 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med Microbiol Immunol 162:9–14.

Underdown BJ, Schiff JM. 1986. Immunoglobulin A: Strategic defense initiative at the mucosal surface. Annu Rev Immunol 4:389–417.

Wright PF, Webster RG. 2001. Orthomyxoviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin AM, Roizman B, Straus SE, editors. Fields virology. 4th edition. Philadelphia: Lippincott Williams & Wilkins. pp. 1533–1579.

Yetter RA, Lehrer S, Ramphal R, Small PA, Jr. 1980. Outcome of influenza infection: Effect of site of initial infection and heterotypic immunity. Infect Immun 29:654–662.