A poorly neutralizing IgG2a/c response elicited by a DNA vaccine protects mice against Japanese encephalitis virus

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We demonstrated previously that immunization with a DNA vaccine expressing the Japanese encephalitis virus (JEV) envelope (E) protein conferred a high level of protection through a poorly neutralizing antibody response. Here, we further investigated the role of the IgG subclass in this antibody-dependent protection using cytokine co-immunization and cytokine-deficient mice. A significant difference in IgG2a/c but not IgG1 was observed between mice that survived or died following a lethal challenge. Correspondingly, the IgG2a/c response and protection increased in IL-4-deficient mice but decreased in IFN-γ-deficient mice, highlighting the importance of IgG2a/c.

INTRODUCTION

Japanese encephalitis virus (JEV; family Flaviviridae) causes severe encephalitis in southern and eastern Asia (for review, see Mackenzie et al., 2004). It is estimated that JEV infection causes 45 000 cases of encephalitis and 10 000 deaths per year (van den Hurk et al., 2009). Even following recovery, ~50% of survivors suffer long-term neurological sequelae (Burke & Leake, 1988). Due to the limited therapeutic approaches, vaccination is the most efficient strategy to control JEV and significant success has been achieved in many Asian countries (Ding et al., 2003; Hoke et al., 1988; Srivastava et al., 2001). Studies in animals and humans have demonstrated that host immunity correlates with the outcome of JEV infection. It is well known that humoral immunity is a major component of protection against JEV infection. Passive transfer of antisera has been shown to provide strong protection against JEV and other flaviviruses (Engle & Diamond, 2003; Hawkes et al., 1988; Kimura-Kuroda & Yasui, 1988; Kreil & Eibl, 1997; Phillpotts et al., 1987). In addition, studies in immunodeficient mice have shown that protection was unchanged in CD8-deficient mice but lost in immunoglobulin-deficient mice (Larena et al., 2011; Pan et al., 2001). These findings suggested antibodies but not CD8 T-cells play an important role in the protective immunity against JEV; however, the protective role of CD8 T-cells and the cytokines they produce, particularly IFN-γ, cannot be completely ruled out, especially in central nervous system (CNS) infection (Larena et al., 2013).

Neutralization by blocking the binding to the viral receptor on host cells is the major mechanism through which antibodies limit virus infection and becomes the primary criterion to evaluate vaccine efficacy (Hombach et al., 2005). Most neutralizing antibodies recognize envelope (E) or pre-membrane protein on the surface of the virion. However, the protection conferred through passive transfer of anti-non-structural protein NS1 antibodies or a poorly neutralizing E-specific mAb suggests that poorly or non-neutralizing antibodies also play a role in the protective mechanism against flaviviruses (Chung et al., 2006; Li et al., 2008). Two supplementary figures are available with the online version of this paper.
1997). In contrast, IgG2a and IgG2c in some mouse strains are regulated by Th1 cytokines IFN-γ and IL-12 (Barr et al., 2009), and become the most efficient subclass at fixing complement and binding to the Fcγ receptor (FcγR) on macrophages and NK cells (Heusser et al., 1977; Kipps et al., 1985; Neuberger & Rajewsky, 1981). FcγR engagement with antibody–antigen immune complexes activates effector cells that destroy virus-infected cells by complement, antibody-dependent cell-mediated cytoxicity (ADCC) or phagocytosis (Clynes et al., 1998; Fossati-Jimack et al., 2000). There are four murine FcγRs, including three activating FcγR I, FcγR IIIA and FcγR IV, and one inhibitory FcγR, FcγRIIb (Ravetch, 2003). The binding affinity to FcγRs also varies among IgG subclasses. The low-affinity FcγR IIIA and the inhibitory FcγRIIb are capable of binding a broad range of IgG subclasses, including IgG1, IgG2a, IgG2b and IgG2c. By contrast, high-affinity FcγRII preferentially binds mouse IgG2a, and FcγRIIb can bind IgG2a, IgG2b and IgG2c with an intermediate to high affinity (Hamaguchi et al., 2006; Nimmerjahn & Ravetch, 2005). Although FcγR and complement have been reported to be involved in the protective mechanism of poorly or non-neutralizing antibodies (Vogt et al., 2011), the importance of the IgG subclass in the protection mediated by poorly or non-neutralizing antibodies is still unclear.

We have developed previously a JEV DNA vaccine based on a plasmid (pE) expressing E protein lacking the signal peptide required for the native conformation. Immunization of pE elicited a poorly neutralizing antibody response but still provided ~90% protection in a mouse model (Chen et al., 1999). We also demonstrated that the protective mechanism was antibody dependent (Pan et al., 2001). In the present study, we examined whether the IgG subclass plays a role in pE-induced protection by using either cytokine co-delivery or cytokine-deficient mice. The results could expand our understanding in the protective mechanism of the JEV vaccine and provide useful information for the development of flavivirus vaccines.

RESULTS

Modulation of IgG class switching and protection by co-delivery of cytokine genes

To address the role of the IgG subclass in the protective mechanism of pE, co-delivery of plasmid encoding cytokine genes, such as IL-4 (pIL-4) or IL-12 (pIL-12), was used to shift IgG class switching to IgG1 and IgG2a or IgG2c. The IgG2a and IgG2c expression patterns vary in different strains of mice, e.g. C57BL/6 mice express IgG2c but not IgG2a (Zhang et al., 2012). Given that IgG2a and IgG2c share 85% amino acid homology (Schreier et al., 1981) and can be detected by the same mAb used in ELISA, the term ‘IgG2a/c’ is used to represent the Th1-biased IgG subclass in this study. C3H/HeN mice were administered pE with pIL-4, pIL-12 or vector pcDNA3 by either gene gun or intramuscular (i.m.) injection. Mice immunized with pcDNA3 alone at the same dose were used as the control. A specific antibody response was detected in all pE-immunized mice but not the pcDNA3 control (Table 1). There was no difference in the E-specific antibody response between i.m.- or gene-gun-immunized mice with co-delivery of pE and pcDNA3. Co-administration with pIL-4 enhanced significantly the antibody response in gene-gun-immunized mice but not i.m.-immunized mice. The neutralizing activity in all pE-immunized mice was below the detection limit (50% of plaque reduction at a 1:10 dilution), as we observed previously (Chen et al., 1999). Eight weeks after immunization, mice received an intraperitoneal (i.p.) challenge and all pcDNA3-immunized mice died within 5–9 days after challenge. Although a comparable IgG response was observed between pE + pcDNA3 gene gun and i.m. immunization, the latter induced an IgG2a/c-dominant response, with the highest IgG2a/c titre among all immunized mice, and provided 100% protection against JEV challenge. Co-delivery with pIL-4 did not alter the IgG2a/c-dominant response, although slight decreases in the IgG2a/c titre and protection (80%) compared with pE + pcDNA3 immunized mice were observed. In contrast to i.m. immunization, mice immunized by gene gun injection developed high IgG1 and low IgG2a/c titres, and showed 80% protection. Co-delivery with pIL-4 by gene gun injection resulted in a significant increase in both IgG1 and IgG2a/c; however, the protection was similar to that induced by pE and pcDNA3 (80%). Compared with pIL-4, co-delivery of pIL-12 suppressed E-specific antibody responses, and resulted in a decrease in both IgG1 and IgG2a/c, and the induced protection was also reduced (40%).

Correlation of E-specific IgG2a/c in mice surviving JEV challenge

To determine whether the IgG2a/c titre correlated with protection, the individual IgG1 and IgG2a/c titres and the outcome following a JEV challenge of C3H/HeN mice immunized with pE combined with pcDNA3, pIL-4 or pIL-12 in the previous experiments were analysed. There was no significant difference in the E-specific IgG1 titre between mice that survived or died following the JEV challenge (Fig. 1a). However, a significant difference ($P=0.0066$ by the Mann–Whitney $t$-test) was observed in E-specific IgG2a/c between mice that survived and mice that died (Fig. 1b).

Loss of IgG2a/c and protection in pE-immunized IFN-γ-deficient mice

We further used IL-4- or IFN-γ-deficient mice to examine the roles of the IgG subclass in pE-induced protection. After gene gun immunization, a comparable antibody...
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**Table 1.** Antibody responses, neutralization titres and protection induced by pE with cytokine genes

Response at week 8 was observed in WT C57BL/6 (B6) mice, IL-4 knockout (KO) and IFN-$\gamma$ KO mice, with IgG titres of 368±319, 361±239 and 491±334 U ml$^{-1}$, respectively (Fig. 2a). Following challenge, the survival rate of B6 mice was 80% ($n=5$; Fig. 2b), similar to previous results in C3H/HeN mice (Table 1). The protection induced by pE was a little but not significantly higher in IL-4 KO mice (100%; $n=7$) than B6. Surprisingly, the protection of pE in IFN-$\gamma$ KO mice was diminished (0%; $n=5$) and significantly lower than B6 mice ($P<0.05$ by the Mantel–Cox test). The time required for the development of clinical symptoms in IFN-$\gamma$ KO mice was 5–8 days, similar to B6 and C3H/HeN mice. We also measured the titres of the IgG subclass in pE-immunized mice. Completely different IgG subclass profiles were observed between IL-4 KO and IFN-$\gamma$ KO mice. An IgG1-dominant response without a detectable level of IgG2a/c was induced in IFN-$\gamma$ KO mice (Fig. 2c). In contrast, an IgG2a/c-dominant response was elicited in IL-4 KO mice and no specific IgG1 was detected. The disappearance of protection and IgG2a/c in IFN-$\gamma$ KO mice suggests that IFN-$\gamma$ and/or IgG2a/c are involved in the pE-elicited protection. The IgG1 titres in B6, IFN-$\gamma$ KO and IL-4 KO mice were 844±35, 508±14 and 0±0, respectively, and the IgG2a/c titres for B6, IFN-$\gamma$ KO and IL-4 KO mice were 320±8, 0±0 and 1470±12, respectively.

**Restoration of IgG2a/c and protection in IFN-$\gamma$-deficient mice by i.m. immunization**

Given that an IgG2a/c-dominant response was induced in C3H/HeN mice by i.m. immunization, we further examined whether IgG2a/c class switching in IFN-$\gamma$-deficient mice could be restored by a Th1-biased i.m. immunization, as seen in Table 1. Following pE immunization, there was no significant difference in E-specific antibody titres between IFN-$\gamma$ KO, IL-4 KO and B6 mice (Fig. 3a). The peak (week 8) IgG titres in IFN-$\gamma$ KO, IL-4 KO and B6 mice were 160±76, 414±312 and 128±66 U ml$^{-1}$, respectively. Interestingly, protection against JEV challenge was restored in i.m.-immunized IFN-$\gamma$ KO mice. The survival rates were 71 ($n=7$), 100 ($n=5$) and 78% ($n=9$) for IFN-$\gamma$ KO, IL-4 KO and B6 mice, respectively (Fig. 3b). In contrast to gene gun immunization, an IgG2a/c-dominant specific antibody response was induced in B6 mice by i.m. immunization. In the IFN-$\gamma$ KO mice, IgG2a/c was also induced and showed a similar titre to IgG1 (Fig. 3c). The IgG2a/c titres were 34±12, 1470±19 and 368±14 for IFN-$\gamma$ KO, IL-4 KO and B6 mice, respectively. In contrast, IgG1 was only detected in IFN-$\gamma$ KO mice (40±27). Similar to the observation in C3H/HeN mice (Fig. 1), a significant correlation of IgG2a/c but not IgG1 with the challenge outcome in C57BL/6 background mice was seen (Fig. S1, available in the online Supplementary Material).

**IgG2a/c-mediated protection failed to protect CNS infection from JEV**

To understand the potential of pE-elicited antibodies to protect CNS infection from JEV, a direct intracranial (i.c.)
challenge was used. We first compared the viral load in the CNS between i.c. and i.p. challenge, and found no difference in the viral RNA concentration after day 3 post-challenge (Fig. S2). To evaluate the pE-induced protection in the CNS, mice immunized with pE by gene gun or i.m. injection, pcDNA3 by i.m. injection, or a sublethal dose of live JEV by i.p. inoculation received an i.c. challenge 8 weeks later. All mice died within 7–8 days post-challenge except live JEV-immunized mice, which showed a 50 % (n=4; Fig. 4a) survival rate, significantly higher than other groups (P<0.005 by the Mantel–Cox test). To further determine whether the protection provided by pE-induced antibodies was prophylactic and/or therapeutic, mice received a passive transfer of pooled sera from pE-immunized mice pre-challenge or 24 h post-challenge. An 84 % (n=6) survival rate was observed in mice that received the sera transfer pre-challenge (Fig. 4b). This was significantly different from the results observed among mice receiving the sera transfer 24 h post-challenge (0 %; n=6; P<0.001 by the Mantel–Cox test).

**DISCUSSION**

We reported previously that antibody responses elicited by the pE DNA vaccine demonstrated poor neutralizing activity, yet still protected mice from JEV infection. Here, we further examined the role of IgG subclasses in the protection mediated by poor neutralizing antibodies. Using either co-delivery with Th1 or Th2 cytokines to modulate IgG class switching, a significant difference in IgG2a/c but not IgG1 titres was observed between mice that survived and mice that died following a lethal challenge (Fig. 1). As the titres of IgG2b and IgG3 were much lower than those of IgG1 and IgG2a/c in pE-immunized C3H/HeN mice (data not shown), IgG2a/c is likely to be associated with the protection induced by pE immunization. In addition, the fact that the IgG2a/c response and protection increased in IL-4-deficient mice but decreased in IFN-γ-deficient mice highlights the important role of IgG2a/c in the protective mechanism. Strong evidence was provided by the restoration of the IgG2a/c response and protection in IFN-γ-deficient mice.
mice by i.m. immunization, which induced a Th1-biased response. Correspondingly, our previous report also demonstrated that passive transfer with the IgG2a/c-dominated sera from mice i.m.-immunized with pE provided higher protection than the IgG1-dominated sera from gene-gun-immunized mice (Pan et al., 2001). The correlation of pE-induced IgG2a/c but not IgG1 with protection suggests that IgG2a/c plays a critical role in the protective mechanism of pE immunization. Similar to our findings, IgG2a/c have been reported to play an important role in protection against other infectious diseases (Baldridge & Buchmeier, 1992; Coutelier et al., 1987; Markine-Goriaynoff & Coutelier, 2002; Schlageter & Kozel, 1990).

Unlike other JEV vaccines that induce neutralizing antibodies, immunization with pE elicited a poorly neutralizing antibody response, possibly due to the misfolding of E without the signal peptide located in the pre-membrane protein region (Konishi & Mason, 1993). Similar findings of protection mediated by poorly or no neutralizing antibodies have also been reported in other viral infections, including dengue virus, alphavirus, influenza virus and West Nile virus (Huber et al., 2001; Kaufman et al., 1989; Schmaljohn et al., 1982; Vogt et al., 2011). Most of the reported protective effects are mediated through FcR-dependent mechanisms. As JEV enters the CNS within 24 h after i.p. challenge in our animal model (Fig. S2), it is unlikely that FcR-dependent ADCC or complement fixation account for the IgG2a/c-mediated protection. Therefore, it is reasonable to speculate that IgG2a/c clears JEV from the periphery through FcR- or complement-receptor-mediated phagocytosis, similar to the findings observed in West Nile virus infection (Chung et al., 2007).

The IgG2a/c class switching observed in IFN-γ-deficient mice after i.m. injection suggested that an IFN-γ-independent pathway was responsible. It has been reported that IgG2a/c class switching could be induced by type I IFNs (Peng et al., 2002). Therefore, we presume that the class switch to IgG2a/c in the IFN-γ-deficient mice was due to

![Fig. 3. Protection and the IgG2a/c response in IFN-γ KO mice were restored by i.m. injection. Cytokine-deficient (IL-4 KO and IFN-γ KO) and C57BL/6 mice (B6) were immunized with 100 μg pE by i.m. injection three times at 3 week intervals. (a) JEV-specific IgG assayed by ELISA is represented as the mean ± s.d. (b) Eight weeks later, immunized mice were challenged with 50 LD_{50} JEV and the percentage of surviving mice is shown. (c) E-specific IgG1 and IgG2a/c titres determined by ELISA are represented as the mean ± s.d.](http://vir.sgmjournals.org)

![Fig. 4. Immunization with pE failed to protect mice from an i.c. challenge. (a) C3H/HeN mice immunized with pE by either i.m. (n=5) or gene gun (n=5) injection, pcDNA3 control (n=5), or live JEV (n=4) were challenged with 500 p.f.u. JEV (~20 LD_{50}) at week 8 by the i.c. route and the survival rate is represented. (b) Groups of C3H/HeN mice (n=6) received a passive transfer with 100 μl sera from pE-, pcDNA3- or live JEV-immunized C3H/HeN mice before (Pre) or after (Post) a lethal i.p. challenge (50 LD_{50}; 3×10^7 p.f.u. of JEV) and the survival rate is indicated.](http://vir.sgmjournals.org)
type I IFNs induced by the pE DNA vaccine. Considering the 100-fold difference in the amount of pE plasmid used in i.m. injection compared with gene gun injection, there is no doubt that more type I IFNs were produced by the innate immune system after the i.m. injection. Although IFN-γ- and IFN-α receptor-deficient mice have been shown to be more vulnerable to flavivirus infection (Shresta et al., 2004, 2006), the loss of protection in pE-immunized IFN-γ-deficient mice cannot be explained simply by the lack of IFN-γ. Given that similar survival times were observed in IFN-γ-deficient and B6 mice, and that entry into the CNS by JEV occurred within 24 h in our challenge model, it is unlikely that the higher and prolonged viral burden in the periphery observed in IFN-γ-deficient mice affected the protection induced by pE. In contrast to IFN-γ-deficient mice, the slightly enhanced protection observed in IL-4-deficient mice suggests that the absence of IL-4 provides some benefit to the pE-induced protection. Given the increase in IFN-γ production detected in IL-4-deficient mice after infection (Smythies et al., 2000), and the upregulation of FcγR I and FcγR IV expression on macrophages by IFN-γ (Nimmerjahn et al., 2005), the higher IFN-γ production in IL-4-deficient mice may induce both IgG2a/c class switching and its activation receptors, thereby amplifying the role of the IgG2a/c subclass in mediating protective responses in vivo similar to previous findings (Pricop et al., 2001).

According to the findings of a passive transfer experiment with a poorly neutralizing mAb (Kimura-Kuroda & Yasui, 1988), the failure of pE immunization to protect against the i.c. challenge was not surprising. In contrast to the poorly neutralizing antibodies in our system, it has been reported that passive transfer with neutralizing antibodies against West Nile virus or JEV provides protection even after the virus has been detected in the brain (Engle & Diamond, 2003; Kimura-Kuroda & Yasui, 1988). Interestingly, partial protection against the i.c. challenge was observed in live JEV-immunized mice, suggesting that neutralizing antibodies and/or cellular immunity induced by the live virus contributes to viral clearance in the CNS.

In this paper, we expanded our previous work to examine the influence of IgG subclasses on the protection against JEV infection. Our findings suggest that IgG2a/c plays an important role in the protection mediated by poorly neutralizing antibodies. Considering that a large part of E-specific antibodies shows poorly or no neutralizing activity after JEV vaccination, our data provide an alternative concept in the development of future vaccines against flavivirus.

**METHODS**

**Viruses and animals.** The JEV Beijing-1 virus strain was used as the antigen for immunoassays, and was amplified by Vero cells in roller-bottle cultures and partially purified by ultrafiltration at the cut-off molecular mass of 300 kDa followed by formalin inactivation. For the challenge experiments, the same strain of live virus was maintained in suckling mouse brains for the preparation of a virus stock, as described previously (Chen et al., 1999). Female C3H/HeN or C57Bl/6 mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, and housed at the Laboratory Animal Center, National Health Research Institutes, Taiwan. C57Bl6/IL4m1 (IL-4 KO) and C57Bl6/IFNγm1 (IFN-γ KO) mice, kindly provided by John Kung (Academia Sinica), were maintained at the animal facility. Animal care was provided in accordance with the guidelines approved by the Animal Committee of the National Health Research Institutes.

**Immunization and challenge.** The plasmid pE encoding the E protein of JEV Beijing-1 strain was described previously (Chen et al., 1999). The cytokine plasmids pIL-4 and pIL-12 have been described elsewhere (Chow et al., 1998). Plasmid DNA was purified from the transformed *Escherichia coli* strain DH5α with Plasmid Giga kits (Qagen) and reconstituted in sterile saline for experimental use. Groups of 6-week-old mice received three immunizations at intervals of 3 weeks. For i.m. injections, both quadriceps muscles of mice were pretreated with 50 μl 10 μM cardiotaxin (Sigma) 1 week earlier and injected bilaterally with 50 μg pE with/without 50 μg cytokine plasmid or control pcDNA3 in each quadriceps muscle (total 100 μg for each plasmid per dose). For gene gun immunization, the animals received three immunizations at 3-week intervals with 1 μg pE with/without 1 μg cytokine plasmid or control pcDNA3 in the abdominal epidermis delivered by a hand-held, helium-driven Helios delivery system (Bio-Rad), at the setting of 500 p.s.i. For i.p. challenge, mice were i.p. inoculated with 50 times the LD50 of Beijing-1 strain JEV (3 × 102 and 5 × 106 p.f.u. for C3H/HeN and C57Bl6/6 strains of mice, respectively), followed by a sham i.c. inoculation with 30 μl PBS, as described previously (Chen et al., 1999). In some experiments, mice received an i.c. challenge by inoculation with 500 p.f.u. Beijing-1 strain JEV (~20 LD50). The health status of the challenged mice was monitored daily for 15 days.

**ELISA.** Sera were obtained by tail bleeding and stored at −20 °C until use. JEV-specific antibodies were measured by ELISA, as described previously (Pan et al., 2001). Briefly, diluted sera were added to microtitre plates coated with formalin-inactivated JEV. After washing, the bound antibodies were detected by HRP-conjugated goat anti-mouse IgG (Chemicon). The colour was developed by adding 2,2'-azino-bis(ethylbenzathiazoline sulfonic acid) (Sigma) and the absorbance was measured. The readings were referenced to a serum standard and the results presented as arbitrary units per millilitre (U ml−1; 1 U ml−1 = 22 ng ml−1), as described previously. The IgG subclasses of E-specific antibodies were also determined using the same protocol, in which the detection antibody was replaced with either biotinylated rat anti-mouse IgG1 (1:1000; BD Pharmingen) or rat anti-mouse IgG2a/c (1:1000; BD Pharmingen; based on the manufacturer’s description, this mAb R19-15 showed a high reactivity to IgG2a and weak cross-reactivity to IgG2c, but not other isotypes), followed by avidin-conjugated HRP (1:2000; BD Pharmingen). End-point titres were defined as the highest serum dilution that resulted in an absorbance value twofold greater than that of the non-immune serum with a cut-off value of 0.05.

**Neutralization assay.** The neutralization test was carried out using the 50% plaque reduction technique with BHK-21 cells. A twofold serial dilution of pooled sera was prepared in PBS with 5% bovine calf serum (BCS). Dilutions were incubated at 56 °C for 30 min to inactivate complement and mixed with equal volumes of infectious JEV in minimal essential medium (MEM) supplemented with 5% BCS to yield a virus–antibody mixture. The mixtures were incubated at 4 °C for 18–21 h and then added 0.2 ml of mixtures containing a estimated 100 p.f.u. to triplicate wells containing confluent monolayers of BHK-21 cells. The plates were incubated at 37 °C for 1 h, then overlaid with 2 ml 1% methylcellulose prepared in MEM supplemented with 5% BCS and incubated at 37 °C with 5% CO2 for
3 days. Plaques were fixed by adding 10% formalin, stained with crystal violet and counted. The neutralizing antibody titre was calculated as the reciprocal of the highest dilution resulting in a 50% reduction of plaques compared with a virus control with no added antibody. A diluted E3 mAb with neutralizing activity (a neutralizing titre of 40) was included as a control.

**Adoptive or passive transfer.** Pooled sera or fresh splenocytes were collected from C3H/HeN donor mice immunized three times with DNA vaccines 7 weeks after priming. Recipient C3H/HeN mice were intravenously inoculated with 100 µl pooled immune sera by retro-orbital cavity injection either 12 h prior to challenge or 24 h after challenge. For adoptive transfer, a single-cell suspension of 2 × 10⁶ freshly prepared splenocytes in 100 µl saline was intravenously inoculated into recipient C3H/HeN mice by retro-orbital cavity injection 12 h prior to challenge.

**Statistic analysis.** Student’s t-test was used to analyse the immunoassay data if not otherwise indicated. The challenge results were analysed using Mantel–Cox survival analysis with GraphPad Prism 5 software.

**ACKNOWLEDGEMENTS**

We thank John Kung (Academia Sinica) for providing the IL-4 KO and IFN-γ KO mice. This study was supported by the National Sciences Council, Taiwan, grant NSC98-2320-B-400-012, to C. H. P.

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