Platycodin D Improves the Immunogenicity of Newcastle Disease Virus-Based Recombinant Avian Influenza Vaccine in Mice

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The development of an effective influenza vaccine is urgently important for controlling outbreaks of the highly pathogenic avian influenza virus (HPAIV) and reducing the impact of pandemics. The use of an adjuvant in such a vaccine can significantly contribute to improve the immunogenicity. To explore a novel and safe adjuvant for improving the potency of influenza vaccines, platycodin D (**1**), a saponin from the root of *Platycodon grandiflorum*, was evaluated for the adjuvant potentials on the specific cellular and humoral immune responses to Newcastle disease virus-based recombinant avian influenza vaccine (rL-H5) in mice. Compound **1** significantly promoted the concanavalin A (Con A)-, the lipopolysaccharide (LPS)-, and the antigen-induced splenocyte proliferation and enhanced the serum antigen-specific IgG, IgG1, IgG2a, and IgG2b antibody titers ($P<0.05$, $P<0.01$, or $P<0.001$) in mice immunized with rL-H5. The mRNA expressions of Th1/Th2 cytokines (IFN-γ and IL-10) and transcription factors (T-bet and GATA-3) in splenocytes were also markedly up-regulated by **1**, compared with the control group immunized with rL-H5 alone ($P<0.01$ or $P<0.001$). In addition, **1** remarkably increased the killing activities of natural killer (NK) cells from splenocytes in the immunized mice ($P<0.05$), which may have important implications for the vaccination against the avian influenza virus. We concluded that **1** could improve the immunogenicity of the rL-H5 vaccine by enhancing both humoral and cellular immune responses in mice, and that **1** is a promising adjuvant for influenza vaccines.

Introduction. – Avian influenza is a worldwide occurring infectious disease of poultry [1]. During the last decade, extensive outbreaks of fowl plague caused by the highly pathogenic avian influenza viruses (HPAIV) of the hemagglutinin (HA) subtypes H5 or H7 combined with various neuraminidase (NA) subtypes occurred in most parts of the world [2][3]. Since the middle of 2005, the outbreak was widely spread by infected wild waterfowls carrying viruses, and more than 50 countries in Russia, Middle East Asia, Africa, and Europe were affected [4].

The HPAIV are highly lethal in poultry and can cause large outbreaks leading to substantial economic loss. Moreover, they can spread directly from poultry to humans, constituting a possible 'pandemic threat' to the human population [5]. Although all H5N1 viruses isolated from humans retain characteristic features of the avian influenza viruses (AIV) [6] and are not currently transmissible among humans, the potential of a pandemic caused by H5N1-HPAIV is increasing [7]. In May 2009, 424 laboratory-

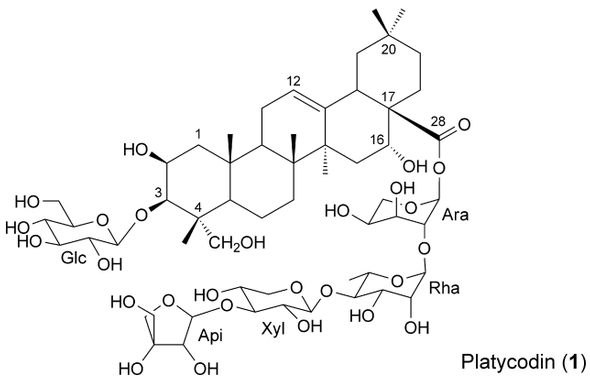
confirmed human H5N1 infections with an approximate 60% case-fatality rate (261 deaths) have been reported to the WHO [8]. To reduce the primary risk of human HPAIV infection, the effective control of avian influenza in poultry is, therefore, an important issue for public health [9].

Vaccination is a cost-effective approach for controlling outbreaks of HPAIV and reducing the impact of pandemics [10]. Since vaccination with inactivated AIV is usually efficacious [11], but requires time-consuming individual administration and interferes with serological and syndrome surveillance of natural AIV infections, numerous attempts have been made to develop subunit, DNA, or vectored vaccines, which give good protection and permit reliable differentiation of infected from vaccinated animals (DIVA strategy) [9]. The subtype-specific protection against HPAIV infections could be achieved by immunization with retrovirus- or baculovirus-expressed HA [12], with plasmid DNA or defective adenoviruses containing HA gene expression cassettes [13–16], and with vectored HA-expressing live virus vaccines based on the fowlpox virus (FPV) [17] or the Newcastle disease virus (NDV) [18–21]. Most of the recombinant vaccines developed so far could support the DIVA strategy, since they fail to induce antibodies against conserved AIV proteins like the nucleoprotein (NP) which are formed during AIV infection and are detectable by ELISA [22]. However, most of the recombinant vaccines are not suitable for mass application, but require repeated individual administration of high amounts of DNA or antigen. On the other hand, the AIV-specific efficacy of vectored NDV and FPV vaccines is significantly reduced in animals, which had been previously immunized with conventional vaccines against ND or FP [23][24]. Although FPV- and NDV-based AIV vaccines are already used in practice, the clinical studies revealed that their immunity in chickens was extremely low.

A number of options have been proposed for improving immune responses to current vaccines; among these is the addition of an adjuvant to the vaccines [25][26]. Some adjuvants, such as MF59 [27][28], aluminum compounds (Alum) [29], and QS-21 [30], have been proposed to improve the immunogenicity of H5N1 vaccines. A major limitation in the use of alum for a variety of vaccines is that it fails to induce Th1 immune responses and stimulates cell-mediated immune responses [31]. Although MF59-induced optimal hemagglutination-inhibition titers as a single adjuvant and also induced potent T-cell responses, only the addition of CpG to MF59 allowed the induction of a potent Th1 response [32]. QS-21 is a Th1 adjuvant that enhances both humoral and cell-mediated immune responses. However, it has serious drawbacks such as high toxicity, a high hemolytic effect, instability in the aqueous phase, and commercial unavailability [33]. This has led to additional research for alternative adjuvants.

Platycodin D (**1**) is a less hemolytic and more stable saponin isolated from the root of *Platycodon grandiflorum*. Although **1** has been shown to be a potent adjuvant of specific immune responses to ovalbumin [34] and recombinant hepatitis B surface antigen [35], the magnitude and quality of immune responses induced by an adjuvant may depend on the nature of the antigen. Thus, the present study was designed to determine whether **1** used as an adjuvant with Newcastle disease virus-based recombinant avian influenza vaccine (rL-H5) could improve the induction of a cellular immune response and at the same time increase the titers of the protective antibodies

IgG2a and IgG2b in mice, to provide a novel and safe adjuvant for enhancing the potency of influenza vaccines.



Platycodin (**1**)

Results. – *Effects of* **1** *on Splenocyte Proliferation in Mice Immunized with rL-H5*. The effects of **1** on the mitogen- and H5Ag-stimulated splenocyte proliferation in mice immunized with rL-H5 are shown in *Fig. 1*. The concanavalin A (Con A)-stimulated splenocyte proliferation in mice immunized with rL-H5 and **1** at the doses of 75 and 100 μg or with rL-H5 and Quil A (= mixture of triterpene saponins from the bark of *Quillaja saponaria*) at 25 μg was significantly higher than that in the rL-H5 alone group and the rL-H5/Alum group ($P<0.01$). Also the lipopolysaccharide (LPS)- and the H5Ag-induced splenocyte proliferation in mice immunized with rL-H5/**1** and rL-H5/Quil A was higher than that observed for the mice immunized with rL-H5 alone and rL-H5/Alum ($P<0.05$, $P<0.01$, or $P<0.001$). However, no significant differences ($P>0.05$) were observed between the rL-H5 alone group and the rL-H5/Alum group. These data indicate that **1** was more effective than Alum in inducing a strong activation potential of T and B cells in mice immunized with rL-H5.

Effects of **1** *on Natural Killer (NK) Cell Activity in Mice Immunized with rL-H5*. The effects of **1** on the NK cell activity in mice immunized with rL-H5 are shown in *Fig. 2*. The killing activity of NK cells in mice immunized with rL-H5 was significantly enhanced ($P<0.05$ or $P<0.01$) by **1** at 50, 75, and 100 μg as well as by Quil A at 25 μg. There were, however, no significant differences ($P>0.05$) in the killing activity of NK cells between mice immunized with rL-H5 alone and rL-H5/Alum. These findings indicate that **1** could promote the lytic activity of NK cells in mice immunized with rL-H5.

Effects of **1** *on the Antigen-Specific Serum Antibody Response*. The effects of **1** on the H5Ag-specific humoral immune responses in mice immunized with rL-H5 are shown in *Fig. 3*. The immunization of the mice with rL-H5 alone induced only low levels of H5Ag-specific total IgG antibody titers. However, the addition of Quil A, Alum, or **1** to rL-H5 resulted in a significant increase in the total IgG antibody titers ($P<0.05$, $P<0.01$, or $P<0.001$). We then assayed the serum antibody isotypes in the immunized mice. The H5Ag-specific IgG1 titers were significantly increased by Quil A, Alum, and **1**, compared with the rL-H5 alone group ($P<0.01$ or $P<0.001$). Regarding

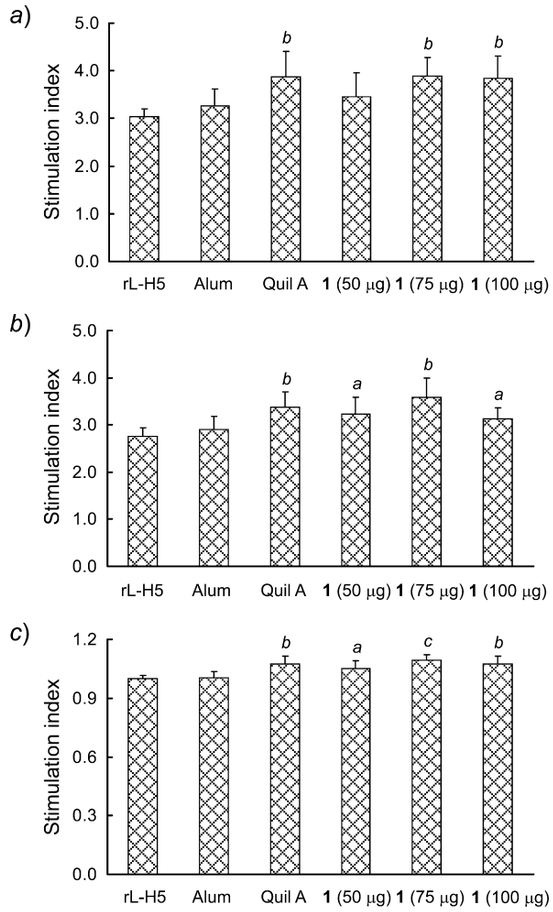


Fig. 1. *Effects of Alum, Quil A, and* **1** *on* a) *Con A-,* b) *LPS-, and* c) *H5Ag-stimulated splenocyte proliferation in mice immunized with rL-H5.* Groups of five mice were immunized *sc* with rL-H5 (10^6 EID_{50}/dose) alone or in combination with Alum (200 μg), Quil A (25 μg), or **1** (50, 75, or 100 μg) on days 1 and 15. Splenocyte proliferation was measured by the MTT assay and shown as a stimulation index (see *Exper. Part*). The values are presented as mean ± SD ($n=5$). Significant differences compared to the rL-H5 and the rL-H5 + Alum groups: $P<0.05$ (*a*), $P<0.01$ (*b*), $P<0.001$ (*c*).

the H5Ag-specific IgG2a and IgG2b titers, they were significantly higher in mice immunized with rL-H5/Quil A and rL-H5/**1** than in mice immunized with rL-H5 alone ($P<0.05$, $P<0.01$, or $P<0.001$). Moreover, the IgG2a and IgG2b antibody titers in the mice immunized with rL-H5/**1** (75 μg) were higher than those in the Alum-treated mice. There were, however, no significant differences ($P>0.05$) in the IgG2a and IgG2b antibody levels between mice immunized with rL-H5 alone and rL-H5/Alum. These findings indicate that **1** significantly enhanced the H5Ag-specific serum antibody

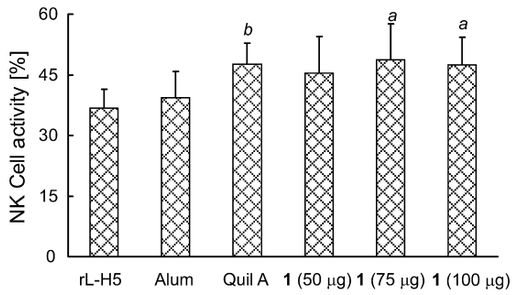


Fig. 2. *Effects of Alum, Quil A, and* **1** *on NK cell activity in mice immunized with rL-H5*. Groups of five mice were immunized *sc* with rL-H5 (10^6 EID_{50}/dose) alone or in combination with Alum (200 μg), Quil A (25 μg), or **1** (50, 75, or 100 μg) on days 1 and 15. Splenocytes were assayed for NK cell activity by the MTT assay (see *Exper. Part*). The values are presented as mean ± SD ($n=5$). Significant differences compared to the rL-H5 and the rL-H5 + Alum groups: $P<0.05$ (*a*), $P<0.01$ (*b*).

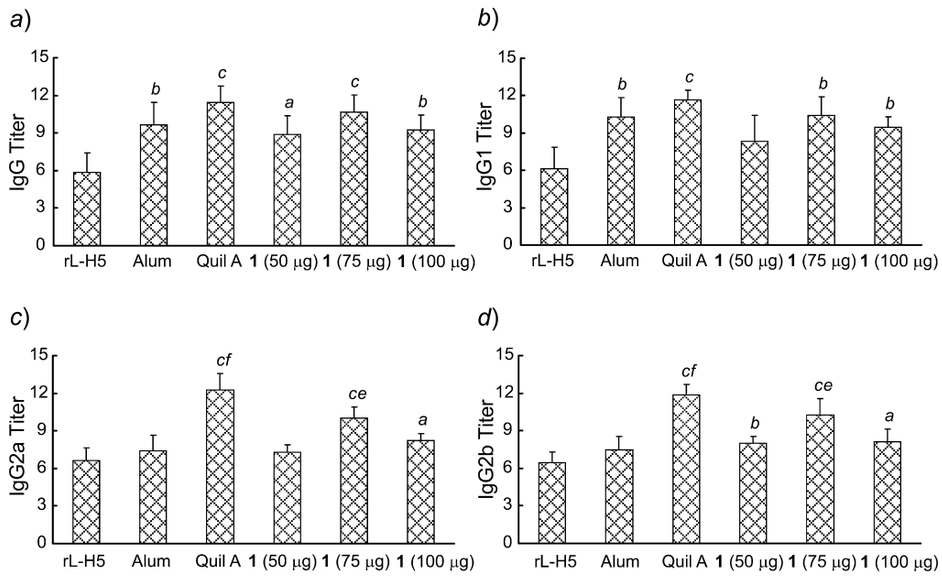


Fig. 3. *Effects of Alum, Quil A, and* **1** *on H5Ag-specific* a) *IgG,* b) *IgG1,* c) *IgG2a, and* d) *IgG2b antibody titers in mice immunized with rL-H5*. Groups of five mice were immunized *sc* with rL-H5 (10^6 EID_{50}/dose) alone or in combination with Alum (200 μg), Quil A (25 μg), or **1** (50, 75, or 100 μg) on days 1 and 15. The serum H5Ag-specific IgG, IgG1, IgG2a, and IgG2b antibody titers were measured by an indirect ELISA test (see *Exper. Part*). The results are presented as mean ± SD ($n=5$). Significant differences compared to the rL-H5 group: $P<0.05$ (*a*), $P<0.01$ (*b*), $P<0.001$ (*c*). Significant differences compared to the rL-H5 + Alum group: $P<0.01$ (*e*), $P<0.001$ (*f*).

production in mice immunized with rL-H5. We further calculated the IgG1/IgG2a and IgG1/IgG2b ratios to evaluate the balance of the Th1- and Th2-type immune responses induced by the adjuvants. The mice immunized with rL-H5/Alum had IgG1/IgG2a and

IgG1/IgG2b ratios of 1.40 and 1.39, respectively, indicative of a preponderant Th2-type immune response. The ratios of the mice immunized with rL-H5/**1** (75 μg) were 1.03 and 1.01, respectively, indicating a balanced Th1/Th2 immune response similar to the one of the mice immunized with rL-H5/Quil A that had ratios of 0.95 and 0.98, respectively.

Effects of **1** *on the mRNA Expression of Cytokines and Transcription Factors.* The effects of **1** on the mRNA expression of the Th1 (IFN-γ) and Th2 (IL-10) cytokines and the transcription factors T-bet and GATA-3 in splenocytes from mice immunized with rL-H5 was measured by RT-PCR using specific primers (*Table 1*), and the results are shown in *Fig. 4* and *Table 2*. Quil A (25 μg) and **1** (75 μg) not only increased the mRNA expression of the Th2 cytokine IL-10 and the transcription factor GATA-3 ($P<0.01$ or $P<0.001$), but also enhanced that of the Th1 cytokine IFN-γ and the transcription factor T-bet ($P<0.01$ or $P<0.001$) in splenocytes from the immunized mice. The IL-10 and GATA-3 mRNA expressions in the immunized mice were also increased by Alum ($P<0.01$ or $P<0.001$). There were, however, no significant differences ($P>0.05$) in the IFN-γ and T-bet mRNA expressions between mice immunized with rL-H5 alone and rL-H5/Alum. Hence, **1** up-regulated the gene expression of Th1 and Th2 cytokines as well as of the transcription factors T-bet and GATA-3 in splenocytes from mice immunized with rL-H5.

Table 1. *Sequences of the Specific Primers Used for RT-PCR*

Gene	Primer sequence	Product size [bp]
GAPDH[a])	5′-CCCACAGTAAATTCAACGGCAC-3′ 5′-CATTGGGGTTAGGAACACGGA-3′	570
IFN-γ	5′-TGAACGCTACACACTGCATCTTGG-3′ 5′-CGACTCCTTTTCCGCTTCCTGAG-3′	459
IL-10	5′-CCAGTTTTACCTGGTAGAAGTGATG-3′ 5′-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′	324
T-bet	5′AACCAGTATCCTGTTCCCAGC-3′ 5′-TGTCGCCACTGGAAGGATAG-3′	436
GATA-3	5′-GAAGGCATCCAGACCCGAAAC-3′ 5′-ACCCATGGCGGTGACCATGC-3′	255

[a]) GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Discussion. – The influenza virus stimulates an antiviral response in B and T lymphocytes, resulting in humoral and cell-mediated immunity, respectively. The response to the influenza viruses is the result of restimulation of a previously primed response through exposure to natural infection or prior vaccination. Virus-activated T cells, through a variety of cytokine mediators, stimulate B cells to differentiate and produce antibodies that are specific for the strains of virus contained in the vaccine [36]. These specific antibodies bind to the surface glycoproteins (HA and NA) to neutralize the viral particle. In contrast to B cells that have a subtype- and strain-specific response, the antigenic determinants of the T cell response are more conserved across the different strains of influenza. T Cell recognition and responses to influenza do not degrade with antigenic drift [37–39]. In other words, antibody responses are

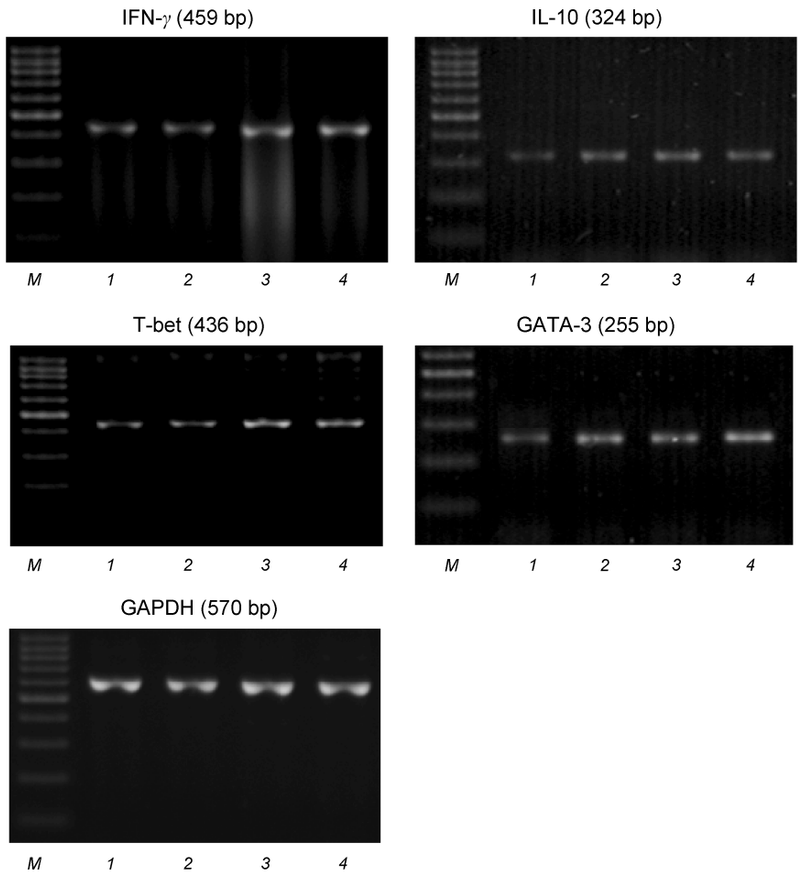


Fig. 4. *Effects of Alum, Quil A, and* **1** *on the mRNA expression of GAPDH, cytokines, and transcription factors in splenocytes from mice immunized with rL-H5.* Groups of five mice were immunized *sc* with rL-H5 (10^6 EID_{50}/dose) alone or in combination with Alum (200 μg), Quil A (25 μg), or **1** (75 μg) on days 1 and 15. The mRNA expression of GAPDH, IFN-γ, IL-10, T-bet, and GATA-3 was analyzed by RT-PCR using specific primers (see *Table 1* and *Exper. Part*). The amplified cDNAs were resolved on a 1.5% agarose gel and visualized by *GoldView* staining. *Lane M*: DNA marker, *Lane 1*: rL-H5, *Lane 2*: rL-H5 + Alum, *Lane 3*: rL-H5 + Quil A, *Lane 4*: rL-H5 + **1** (75 μg).

strain-specific, while T cell responses are cross-reactive. Thus, an optimal influenza vaccine should be capable of eliciting the highest possible antibody titers and enhancing the cellular immune response [32][40]. However, particularly troubling is the difficulty in generating a robust immune response against HPAIV by the use of traditional vaccine approaches [41][42]. The current study was undertaken to evaluate the adjuvant potential of **1** to enhance both humoral as well as cell-mediated immune responses in mice immunized against rL-H5.

Table 2. *Effects of Alum, Quil A, and* **1** *on the mRNA Expression of Cytokines and Transcription Factors in Splenocytes from Mice Immunized with rL-H5*. The mRNA expression was analyzed by RT-PCR using specific primers (see *Table 1* and *Exper. Part*). The amplified cDNAs were resolved on 1.5% agarose gel and visualized by *GoldView* staining. The resulting amounts of mRNA expression were normalized by dividing by the amount of GAPDH gene expression and presented as mean ± SD ($n=3$).

Gene	Groups			
	rL-H5	rL-H5 + Alum (200 μg)	rL-H5 + Quil A (25 μg)	rL-H5 + **1** (75 μg)
IFN-γ	0.25 ± 0.01	0.28 ± 0.01	0.56 ± 0.06 (*c*)[a])	0.50 ± 0.01 (*c*)
T-bet	0.42 ± 0.03	0.50 ± 0.09	0.72 ± 0.08 (*b*)	0.58 ± 0.05 (*b*)
IL-10	0.20 ± 0.03	0.57 ± 0.05 (*c*)	0.57 ± 0.04 (*c*)	0.42 ± 0.03 (*c*)
GATA-3	0.33 ± 0.03	0.65 ± 0.06 (*b*)	0.53 ± 0.09 (*b*)	0.55 ± 0.13 (*b*)

[a]) Significant differences with the rL-H5 alone group were designated as (*b*) $P<0.01$ and (*c*) $P<0.001$.

The cellular immune response plays an important role in the host response to influenza by limiting the virus replication by accelerating the clearance of virus-infected cells [43] and by generating both humoral and cytotoxic T lymphocyte (CTL)-mediated responses to influenza vaccination [44][45]. The capacity to elicit an effective T and B lymphocyte immunity can be shown by the stimulation of the lymphocyte proliferation response. It is generally known that Con A stimulates T cell and LPS B cell proliferation. The proliferation assay showed that **1** could significantly promote the Con A-, LPS-, and H5Ag-stimulated splenocyte proliferation in mice immunized with rL-H5. There were, however, no significant differences in the splenocyte proliferation between the rL-H5 alone group and the rL-H5/Alum group. The results indicated that **1** could increase the activation potential of T and B lymphocytes and induce the humoral and the cell-mediated immune response in mice immunized with rL-H5.

NK Cells and CTL represent two major populations of cytotoxic lymphocytes and are the first line of innate defense against cancer cells and virus-infected cells [46–48]. Studies have revealed that the CTL response confers cross-protection from lethal challenge with heterologous influenza viruses [49]. With spontaneous cell-mediated cytotoxicity, NK cells are functionally similar to CTLs. NK cells are able to deliver a response immediately after recognizing specific signals, including stress signals, 'danger' signals, or signals from molecules of foreign origin, and to react against and destroy target cells without prior sensitization to it [50]. The NK cell-activity assay is a routine method for the analysis of the cellular immune response *in vitro*. In this investigation, addition of Alum to rL-H5 did not result in an increase in the killing activity of NK cells in the immunized mice. In contrast, **1** significantly enhanced the lytic activity of NK cells in mice immunized with rL-H5 ($P<0.05$), suggesting that the concomitant administration of **1** with rL-H5 could help to improve the cytolytic activities against AIV.

We also evaluated the adjuvant activities of **1** on the humoral immune responses to rL-H5. The addition of **1** to rL-H5 resulted in an increase in IgG antibody responses to levels induced by Alum and Quil A. In addition to their neutralizing properties, antibodies can mediate host-effector functions and facilitate the removal of a pathogen from a host. Specifically, the Fc portion of IgG2a interacts with complement components [51] and activatory Fc receptors [52] with a high affinity. This interaction

can efficiently activate Fc receptor-mediated effector functions, which include the stimulation of antibody-dependent, cell-mediated cytotoxicity [53] and opsonophagocytosis by macrophages [54]. The latter has been shown to contribute to the clearance of influenza viruses from infected hosts [55]. Stimulation of IgG2a antibodies has been associated with increased efficacy of influenza vaccination [56–58]. Additionally, monoclonal antibodies of the IgG2a isotype are more efficient at clearing influenza virus infections than monoclonal antibodies of the IgG1 isotype, displaying similar antigenic specificities [59][60]. Therefore, we further evaluated the effect of **1** on H5Ag-specific IgG1, IgG2a, and IgG2b antibody responses to rL-H5. In our studies, while rL-H5 alone induced low levels of specific IgG1, IgG2a, and IgG2b antibodies, the concomitant administration of **1** with rL-H5 significantly enhanced these antibody isotype titers. Moreover, the IgG1/IgG2a and IgG1/IgG2b ratios in the mice immunized with rL-H5/**1** (75 μg) were 1.03 and 1.01, respectively, indicating that **1** modulated the quality of the immune responses and elicited a balanced Th1/Th2 immune response to rL-H5.

To elucidate the mechanism responsible for the efficacy of **1** on the Th1 and Th2 immune responses, we determined the mRNA expression of IFN-γ, the archetypal Th1 cytokine, and IL-10, the signature Th2 cytokine, in splenocytes from immunized mice by RT-PCR. Compound **1** not only enhanced the mRNA expression of IL-10, but also increased that of IFN-γ. Furthermore, cytokine-mRNA levels were for the most part transcriptionally regulated and selective expression of T-bet and GATA-3 was correlated with cytokine gene and protein expression [61]. Therefore, we measured the T-bet and GATA-3 gene expression in splenocytes from mice immunized with rL-H5, and found that **1** also up-regulated the mRNA expression of T-bet and GATA-3. The high mRNA expression of Th1 cytokine IFN-γ and transcription factor T-bet in splenocytes was consistent with the high IgG2a and IgG2b titers, while the high mRNA expression of Th2 cytokine IL-10 and transcription factor GATA-3 was consistent with the high levels of IgG1 antibodies detected in the rL-H5/**1** groups. Together, these results further confirmed that **1** promoted a balanced Th1/Th2 type immune response to rL-H5 and suggested that the effects of **1** on Th1 and Th2 immune responses may result, at least in part, from the regulation of the mRNA expression of the cytokines and transcription factors.

In conclusion, **1** could improve the immunogenicity of rL-H5 vaccine by enhancing both humoral and cellular immune responses in mice. Compound **1** enhanced the magnitude of the immune responses, and, most importantly, it modulated the quality of the immune responses resulting in a balanced Th1/Th2 immunity. Hence, **1** may be a safe and efficient candidate as adjuvant for avian influenza vaccines.

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Experimental Part

General. Newcastle disease virus (NDV)-based recombinant influenza vaccine (rL-H5), recombinant NDV expressing a H5-subtype avian influenza virus (AIV) hemagglutinin (HA) constructed by reverse genetics, and H5 subtype AIV hemagglutination inhibition detecting antigen (H5Ag) were purchased from the *Harbin Weike Biotechnology Development Company* (Heilongjiang, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), concanavalin A, lipopolysaccharide, *RPMI 1640* medium, and rabbit anti-mouse IgG peroxidase conjugate were purchased from *Sigma Chemical Co.* (Saint Louis, Missouri, USA). Goat anti-mouse IgG1, IgG2a, and IgG2b peroxidase conjugate were obtained from *Southern Biotech. Assoc.* (Birmingham, AL, USA), and Quil A was kindly provided by *Brenntag Nordic A/S* (Denmark). *Trizol* was from *Invitrogen* (USA), *RevertAid*™ M-MuLV reverse transcriptase from *Fermentas* (USA), diethylpyrocarbonate (DEPC) and ribonuclease inhibitor from *Biobasic* (Canada), and oligo(dT)$_{18}$ and Taq DNA polymerase from *Sangon* (China). Fetal calf serum (FCS) was provided by *Hangzhou Sijiqing Corp.*, and aluminum hydroxide gel (Alum) was purchased from *Zhejiang Wanma Pharm Co. Ltd.* (Zhejiang, China).

Platycodin D (= *Platycodigenin 3*-O-*β*-D-*Glucopyranosyl-28*-O-*[β*-D-*apiofuranosyl-(1→3)-β*-D-*xylopyranosyl-(1→4)-α*-L-*rhamnopyranosyl-(1→2)-α*-L-*arabinopyranoside]*; **1**). Platycodin D ($C_{57}H_{92}O_{28}$, 1224.5775) has previously been isolated from the roots of *P. grandiflorum*, and its structure has been elucidated by spectroscopic analysis including 2D-NMR spectroscopy and HR-ESI-MS [34]. The purity of **1** was determined to be 99.09% by HPLC. The stock soln. of **1** (4 mg/ml) in 0.89% saline was sterilized by filtration (0.22-μm *Millipore* filter) and analyzed for its endotoxin level by a gel-clot *Limulus* amebocyte lysate assay. The endotoxin level in the stock soln. was less than 0.5 endotoxin units (EU)/ml.

Experimental Animals. Female ICR mice (Grade II, 5 weeks old) weighing 18–22 g were purchased from the *Zhejiang Experimental Animal Center* (Hangzhou, China; certificate No. 22-2001001) and acclimatized for 1 week prior to use. Rodent laboratory chow and tap water were provided *ad libitum*, and the mice were maintained under controlled conditions at a $24 \pm 1°$, $50 \pm 10\%$ rel. humidity, and a 12-h light/12-h dark cycle. All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals and the guidelines established by the Institute for Experimental Animals of the Zhejiang University, and they were approved by the University Committee for Animal Experiments.

Immunization. Six-week-old ICR mice were divided into seven groups, consisting of five mice each. The animals were immunized subcutaneously (*sc*) on day one with rL-H5 (10^6 EID_{50}/dose) alone or in combination with Alum (200 μg), Quil A (25 μg), or **1** (50, 75, or 100 μg) in 0.2 ml saline. Saline-treated animals were included as controls. A boosting injection was given 2 weeks later. Sera and splenocytes were collected two weeks after the second immunization for the determination of the specific antibody and cellular immune responses to rL-H5.

Splenocyte Proliferation Assay. Spleen collected from the immunized mice under aseptic conditions, in *Hank*'s balanced salt soln. (HBSS, *Sigma*), was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension. The erythrocytes were lysed with NH_4Cl (0.8%, *w*/*v*). After centrifugation (380*g* at 4° for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium (*RPMI 1640* supplemented with 12 mM *HEPES* buffer (pH 7.1), 0.05 mM 2-sulfanylethanol, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% FCS). The cell numbers were counted with a hemocytometer by the trypan blue dye exclusion technique, and the cell viability exceeded 95%. Splenocytes were seeded into four wells of a 96-well flatbottom microtiter plate (*Nunc*) at 5×10^6 cells/ml in 100 μl complete medium, and Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), H5Ag (final concentration 0.125 hemagglutinating units (HAU)/ml), or medium were added to give a final vol. of 200 μl. After incubation at 37° in a humidified atmosphere with 5% CO_2 for 44 h, 50 μl of MTT soln. (2 mg/ml) were added to each well. The plates were incubated for another 4 h, centrifuged at 1400*g* for 5 min, and the untransformed MTT was removed carefully by pipetting. To each well, DMSO (200 μl) was added, and after 15 min shaking, the absorbance was determined with an ELISA reader at 570 nm with a 630-nm reference. The stimulation index was calculated by dividing the absorbance of the mitogen-cultures by the absorbance of the non-stimulated cultures.

Assay of Natural Killer (NK) *Cell Activity.* The activity of the NK cells in the splenocytes from immnunized mice was determined in K562 target cells. Human leukemia K562 cell lines sensitive to NK cells were purchased from the *Institute of Cell Biology, Chinese Academy of Sciences*, Shanghai, China. They were maintained in the logarithmic phase of growth in *RPMI 1640* medium supplemented with 2 mM L-glutamine (*Sigma*), penicillin (100 IU/ml; *Sigma*), streptomycin (100 μg/ml; *Sigma*), and 10% FCS at 37° under humidified air with 5% CO_2.

The K562 target cells were seeded in 96-well U-bottom microtiter plates at 2×10^4 cells/well in *RPMI 1640* complete medium. Splenocytes prepared as described above (effector cells) were added at 1×10^6 cells/well to give an effector/target ratio of 50 : 1. The plates were then incubated for 20 h at 37° in a 5% CO_2 atmosphere. 50 μl of MTT soln. (2 mg/ml) were added to each well, and the plate was incubated for another 4 h and then subjected to the MTT assay. Three kinds of control measurements were performed, *viz.*, target-cell controls, blank controls, and effector-cell controls. The NK cell activity was calculated with *Eqn. 1*:

$$\text{NK activity } [\%] = (OD_T - (OD_S - OD_E))/OD_T \times 100 \quad (1)$$

where OD_T, OD_S, and OD_E are the optical densities of the target-cell control, the test sample, and the effector-cell control, resp.

Measurement of H5Ag-Specific Antibody. H5Ag-Specific IgG, IgG1, IgG2a, and IgG2b antibodies in the serum were detected by an indirect ELISA. The 96-well microplates were coated with 100 μl H5Ag soln. (1 HAU/ml in 50 mM carbonate buffer, pH 9.6) for 24 h at 4°. The wells were washed three times with PBS containing 0.05% (*v*/*v*) *Tween 20* (PBS/*Tween*) and then blocked with 5% FCS/PBS at 37° for 2 h. After three washings with PBS, 100 μl of a series of diluted sera samples or 0.5% FCS/PBS as control were added to triplicate wells. The plates were then incubated for 2 h at 37°, followed by washing (3 × PBS). Aliquots of horseradish peroxidase-conjugated antibody against IgG, IgG1, IgG2a, or IgG2b were added to the wells, and the plates were incubated for 2 h at 37°. After washing, the peroxidase activity was determined as follows. After the addition of 100 μl of substrate soln. (10 mg benzene-1,2-diamine and 37.5 μl of 30% H_2O_2 in 25 ml of 0.1M citrate–phosphate buffer pH 5.0) to each well, the plate was incubated for 10 min at 37°, and the enzyme reaction was stopped by adding 50 μl/well of an aq. 2N H_2SO_4 soln. The optical density (*OD*) was measured with an ELISA reader at 490 nm with a 595-nm reference. Data were expressed as the mean *OD* value of the samples minus the mean *OD* value of the blank control. When sets of serum samples have been subjected to within and between group comparisons, ELISA assays were performed on the same day for all samples.

Measurement of Cytokine and Transcription Factor Gene Expression by RT-PCR. Splenocytes from immunized mice (1 ml in complete medium), prepared as described before, were seeded into flatbottom 24-well microtiter plates at 5×10^6 cells/ml, and Con A (final concentration 6 μg/ml) was added to give a final vol. of 2 ml (triplicate wells). After incubation of the plates at 37° in a 5% CO_2 atmosphere for 16 h, the cells were harvested by centrifugation (380*g* at 4° for 10 min), washed with ice-cold PBS, and subjected to RNA extraction. Splenocytes were lysed in 0.8 ml of *Trizol* reagent, and the total RNA was isolated according to the manufacturer's protocol. The concentration of total RNA was quantified by determining the optical density at 260 nm. The reverse transcription was performed by mixing 2 μg of RNA with 0.5 μg oligo$(dT)_{18}$ primer in a sterile tube. Nuclease-free H_2O was added to give a final vol. of 12.5 μl. This mix was incubated at 70° for 5 min and chilled on ice for 2 min. Then, a soln. containing 4 μl of M-MuLV 5 × reaction buffer, 2 μl of 10 mM dNTP and 20 U of ribonuclease inhibitor were added, and the tubes were incubated for 5 min at 37°. After the addition of 200 U of *RevertAid*™ M-MuLV reverse transcriptase, the tubes were incubated for 60 min at 42°. Finally, the reaction was stopped by heating at 70° for 10 min. The samples were stored at −20° until further use.

Amplification was carried out in a total volume of 25 μl containing 0.5 μl (10 μM) of each cytokine- or transcription factor-specific primer (*Table 1*), 2.5 μl of 10 × PCR buffer, 2 μl of $MgCl_2$ (25 mM), 0.5 μl of dNTP (10 mM), 1 μl of transcribed cDNA, and 0.25 μl of Taq DNA polymerase. PCR was performed for 20 (GAPDH), 23 (IFN-γ), 25 (T-bet and GATA-3), or 28 (IL-10) cycles using a *PTC-200* thermal cycler (*MJ Research*, Watertown, MA) with the following program: denaturation at 94° for 1 min, annealing at 55° (T-bet and GATA-3), 57° (GAPDH), or 58° (IFN-γ and IL-10) for 50 s, and elongation at 72° for

1 min. Semi-quant. RT-PCR was performed using GAPDH as an internal control to normalize the gene expression for the PCR templates. The PCR products were resolved on a 1.5% (*w*/*v*) agarose gel, and the amplified bands were visualized using ImmageMaster VDS software (*Pharmacia Biotech*, USA) after staining with *GoldView*. The size of the amplified fragments was determined by comparison with a standard DNA marker.

Statistical Analysis. The data were expressed as mean ± standard deviations (SD) and examined for their statistical significance of difference with ANOVA and the post-hoc test. P Values < 0.05 were considered to be statistically significant.

REFERENCES

[1] D. J. Alexander, *Vaccine* **2007**, *25*, 5637.
[2] I. Capua, D. J. Alexander, *Biosci. Rep.* **2007**, *27*, 359.
[3] I. Capua, S. Marangon, *Vaccine* **2007**, *25*, 5645.
[4] B. Olsen, V. J. Munster, A. Wallensten, J. Waldenström, A. D. Osterhaus, R. A. M. Fouchier, *Science* **2006**, *312*, 384.
[5] J. S. Malik Peiris, M. D. de Jong, Y. Guan, *Clin. Microbiol. Rev.* **2007**, *20*, 243.
[6] The World Health Organization Global Influenza Program Surveillance Network, *Emerg. Infect. Dis.* **2005**, *11*, 1515.
[7] A. Ninomiya, M. Imai, M. Tashiro, T. Odagiri, *Vaccine* **2007**, *25*, 3554.
[8] http://www.who.int/csr/disease/avian_influenza/country/en/, May 15, 2009.
[9] S. P. Pavlova, J. Veits, G. M. Keil, T. C. Mettenleiter, W. Fuchs, *Vaccine* **2009**, *27*, 773.
[10] J. A. Van der Goot, G. Koch, M. C. M. de Jong, M. van Boven, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 18141.
[11] G. Tian, S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi, K. Yu, H. Chen, *Virology* **2005**, *341*, 153.
[12] L. A. Hunt, D. W. Brown, H. L. Robinson, C. W. Naeve, R. G. Webster, *J. Virol.* **1988**, *62*, 3014.
[13] H. L. Robinson, L. A. Hunt, R. G. Webster, *Vaccine* **1993**, *11*, 957.
[14] W. Gao, A. C. Soloff, X. Lu, A. Montecalvo, D. C. Nguyen, Y. Matsuoka, P. D. Robbins, D. E. Swayne, R. O. Donis, J. M. Katz, S. M. Barratt-Boyes, A. Gambotto, *J. Virol.* **2006**, *80*, 1959.
[15] K. R. Van Kampen, Z. Shi, P. Gao, J. Zhang, K. W. Foster, D.-T. Chen, D. Marks, C. A. Elmets, D.-C. Tang, *Vaccine* **2005**, *23*, 1029.
[16] H. Toro, D. C. Tang, D. L. Suarez, M. J. Sylte, J. Pfeiffer, K. R. Van Kampen, *Vaccine* **2007**, *25*, 2886.
[17] J. Taylor, R. Weinberg, Y. Kawaoka, R. G. Webster, E. Paoletti, *Vaccine* **1988**, *6*, 504.
[18] D. E. Swayne, D. L. Suarez, S. Schultz-Cherry, T. M. Tumpey, D. J. King, T. Nakaya, P. Palese, A. Garcia-Sastre, *Avian Dis.* **2003**, *47*, 1047.
[19] J. Veits, D. Wiesner, W. Fuchs, B. Hoffmann, H. Granzow, E. Starick, E. Mundt, H. Schirrmeier, T. Mebatsion, T. C. Mettenleiter, A. Römer-Oberdörfer, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8197.
[20] J. M. DiNapoli, L. Yang, A. Suguitan Jr., S. Elankumaran, D. W. Dorward, B. R. Murphy, S. K. Samal, P. L. Collins, A. Bukreyev, *J. Virol.* **2007**, *81*, 11560.
[21] D. E. Swayne, M. Garcia, J. R. Beck, N. Kinney, D. L. Suarez, *Vaccine* **2000**, *18*, 1088.
[22] G. F. de Boer, W. Back, A. D. M. E. Osterhaus, *Arch. Virol.* **1990**, *115*, 47.
[23] E. Starick, O. Werner, H. Schirrmeier, B. Köllner, R. Riebe, E. Mundt, *J. Vet. Med., B* **2006**, *53*, 370.
[24] D. E. Swayne, J. R. Beck, N. Kinney, *Avian Dis.* **2000**, *44*, 132.
[25] S. Sambhara, G. A. Poland, *Lancet* **2007**, *370*, 544.
[26] R. B. Couch, W. A. Keitel, T. R. Cate, *J. Infect. Dis.* 1**997**, *176*, S38.
[27] I. Stephenson, K. G. Nicholson, A. Colegate, A. Podda, J. Wood, E. Ypma, M. Zambon, *Vaccine* **2003**, *21*, 1687.
[28] I. Stephenson, R. Bugarini, K. G. Nicholson, A. Podda, J. M. Wood, M. C. Zambon, J. M. Katz, *J. Infect. Dis.* **2005**, *191*, 1210.
[29] N. Hehme, H. Engelmann, W. Kuenzel, E. Neumeier, R. Saenger, *Virus Res.* **2004**, *103*, 163.
[30] I. Mbawuike, Y. Zang, R. B. Couch, *Vaccine* **2007**, *25*, 3263.
[31] H. Hogen-Esch, *Vaccine* **2002**, *20*, S34.

[32] A. Wack, B. C. Baudner, A. K. Hilbert, I. Manini, S. Nuti, S. Tavarini, H. Scheffczik, M. Ugozzoli, M. Singh, J. Kazzaz, E. Montomoli, G. Del Giudice, R. Rappuoli, D. T. O' Hagan, *Vaccine* **2008**, *26*, 552.
[33] D. C. Waite, E. W. Jacobson, F. A. Ennis, R. Edelman, B. White, R. Kammer, C. Anderson, C. R. Kensil, *Vaccine* **2001**, *19*, 3957.
[34] Y. Xie, Y.-P. Ye, H.-X. Sun, D. Li, *Vaccine* **2008**, *26*, 3452.
[35] Y. Xie, H.-X. Sun, D. Li, *Vaccine* **2009**, *27*, 757.
[36] J.-L. Virelizier, R. Postlethwaite, G. C. Schild, A. C. Allison, *J. Exp. Med.* **1974**, *140*, 1559.
[37] R. B. Effros, P. C. Doherty, W. Gerhard, J. Bennink, *J. Exp. Med.* **1977**, *145*, 557.
[38] G. M. Butchko, R. B. Armstrong, F. A. Ennis, *J. Immunol.* **1978**, *121*, 2381.
[39] S. M. Russell, F. Y. Liew, *Nature* **1979**, *280*, 147.
[40] J. E. McElhaney, *Vaccine* **2005**, *23*, S10.
[41] J. J. Treanor, J. D. Campbell, K. M. Zangwill, T. Rowe, M. Wolff, *N. Engl. J. Med.* **2006**, *354*, 1343.
[42] J. M. Wood, *Philos. Trans. R. Soc. London*, *Ser. B* **2001**, *356*, 1953.
[43] P. Moss, *Dev. Biol.* **2003**, *115*, 31.
[44] J. W. Yewdell, J. R. Bennink, Y. Hosaka, *Science* **1988**, *239*, 637.
[45] C. J. Hackett, L. C. Eisenlohr, *Immunol. Res.* **1990**, *9*, 103.
[46] F. J. Kos, E. G. Engleman, *Immunol. Today* **1996**, *17*, 174.
[47] R. Medzhitov, C. A. Janeway Jr., *Curr. Opin. Immunol.* **1997**, *9*, 4.
[48] L. Moretta, C. Bottino, C. Cantoni, M. C. Mingari, A. Moretta, *Curr. Opin. Pharmacol.* **2001**, *1*, 387.
[49] R. J. Webby, S. Andreansky, J. Stambas, J. E. Rehg, R. G. Webster, P. C. Doherty, S. J. Turner, *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7235.
[50] J. S. Orange, *Nat. Rev. Immunol.* **2008**, *8*, 713.
[51] M. S. Neuberger, K. Rajewsky, *Eur. J. Immunol.* **1981**, *11*, 1012.
[52] J. E. Gessner, H. Heiken, A. Tamm, R. E. Schmidt, *Ann. Hematol.* **1998**, *76*, 231.
[53] T. J. Kipps, P. Parham, J. Punt, L. A. Herzenberg, *J. Exp. Med.* **1985**, *161*, 1.
[54] T. Takai, M. Li, D. Sylvestre, R. Clynes, J. V. Ravetch, *Cell* **1994**, *76*, 519.
[55] V. C. Huber, J. M. Lynch, D. J. Bucher, J. Le, D. W. Metzger, *J. Immunol.* **2001**, *166*, 7381.
[56] B. P. Arulanandam, M. O'Toole, D. W. Metzger, *J. Infect. Dis.* **1999**, *180*, 940.
[57] A.-O. Hovden, R. J. Cox, L. R. Haaheim, *Scand. J. Immunol.* **2005**, *62*, 36.
[58] T. M. Moran, H. Park, A. Fernandez-Sesma, J. L. Schulman, *J. Infect. Dis.* **1999**, *180*, 579.
[59] K. Mozdzanowska, M. Furchner, G. Washko, J. Mozdzanowski, W. Gerhard, *J. Virol.* **1997**, *71*, 4347.
[60] W. Gerhard, K. Mozdzanowska, M. Furchner, G. Washko, K. Maiese, *Immunol. Rev.* **1997**, *159*, 95.
[61] H. Chakir, H. Wang, D. E. Lefebvre, J. Webb, F. W. Scott, *J. Immunol. Methods* **2003**, *278*, 157.

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