RESEARCH PAPER

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Nizatidine, a small molecular compound, enhances killed H5N1 vaccine cell-mediated responses and protects mice from lethal viral challenge

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Abbreviations: Nizatidine, NIZ; Cimetidine, CIM; antigen present cells, APCs; Hemagglutination inhibition, HI

Nizatidine (NIZ), closely related to Cimetidine, is a histamine H2 receptor inverse agonist used primarily as an anti-acid drug. Recent studies showed that this class of compounds may also modulate immune responses. To evaluate adjuvant effects of NIZ on vaccine immune modulation, we formulated NIZ with a H5N1 killed viral antigen and tested in vitro and in vivo. NIZ activated DC maturation and stimulated Th1 and Th2 immune responses to H5N1 vaccine. As a result, it enhanced both antibody and T cell-mediated immune responses. We also observed that a single immunization into C57BL/6 mice blocked IL-10 upregulation and potentiated Th1/Th2 dual polarization. Importantly, the inoculation of H5N1 vaccine with NIZ significantly improved protection of animals from death after challenge and reduced virus loads in the lung tissues. Considering its water-soluble nature, compared with Cimetidine, Nizatidine may be a better choice to use as a vaccine adjuvant.

Introduction

Avian A/H5N1 Influenza A virus has emerged as a cause of human disease in recent years. With sporadic human infections with highly pathogenic H5N1 viruses being reported,[1-3] specific control measures are needed. Vaccination has its advantages over other approaches for limiting potential pandemic influenza outbreaks. Despite the success of introducing vaccination programs in birds using killed virus in a number of countries in Asia, H5N1 viruses continue to circulate in the poultry populations and outbreaks are frequently recorded.[4,5] One of main reasons may be the weak cellular response to vaccination that might otherwise help the inactivated vaccine to become a sterilizing vaccine.[6,7] In particular, the induction of broad cytotoxic T lymphocyte responses against H5N1 infections may be necessary.[8,9]

Adjuvants have been proved to enhance cellular immune responses.[10-12] However, mineral oil, alum and MF59 are the few adjuvants allowed for use in influenza vaccines[10,13,14] and they tend to favor stimulation of humoral immunity rather than the cellular response. In consequence, newer and more potent adjuvants for influenza vaccines that can robustly stimulate both human and cellular immune responses are much in demand.

Nizatidine (NIZ) is a histamine H2-receptor antagonist used primarily to treat stomach ulcers and gastro-esophageal reflux disease.[15,16] Recent studies showed that H2-receptor antagonists such as cimetidine (CIM) could modulate immune responses[17-19] and be used as vaccine adjuvants.[20,21] However, the difficulty of dissolving compounds such as CIM in aqueous solution is an obstacle to their acceptance and wide usefulness as adjuvants. Nizatidine, on the other hand, has an excellent water solubility and lower toxicity, which makes it a good choice for evaluation as a vaccine adjuvant. In the present study, we analyzed its potential usefulness as an adjuvant for H5N1 vaccine.

Results

Nizatidine enhances APC activation in vitro

NIZ is an inverse agonist for the histamine H2 receptor, which in turn signals via the PI3K-Akt pathway in APCs to activate the co-stimulation signal. To determine a direct effect of NIZ on APCs, we treated cultured DC line DC2.4 and macrophage line RAW264.7 with NIZ and CIM (a positive control) and examined changes in the expression of functional markers including MHC II, CD80, CD86, and CD40. The levels of MHC II and CD40 were increased in DC2.4 cell line in the presence of both NIZ

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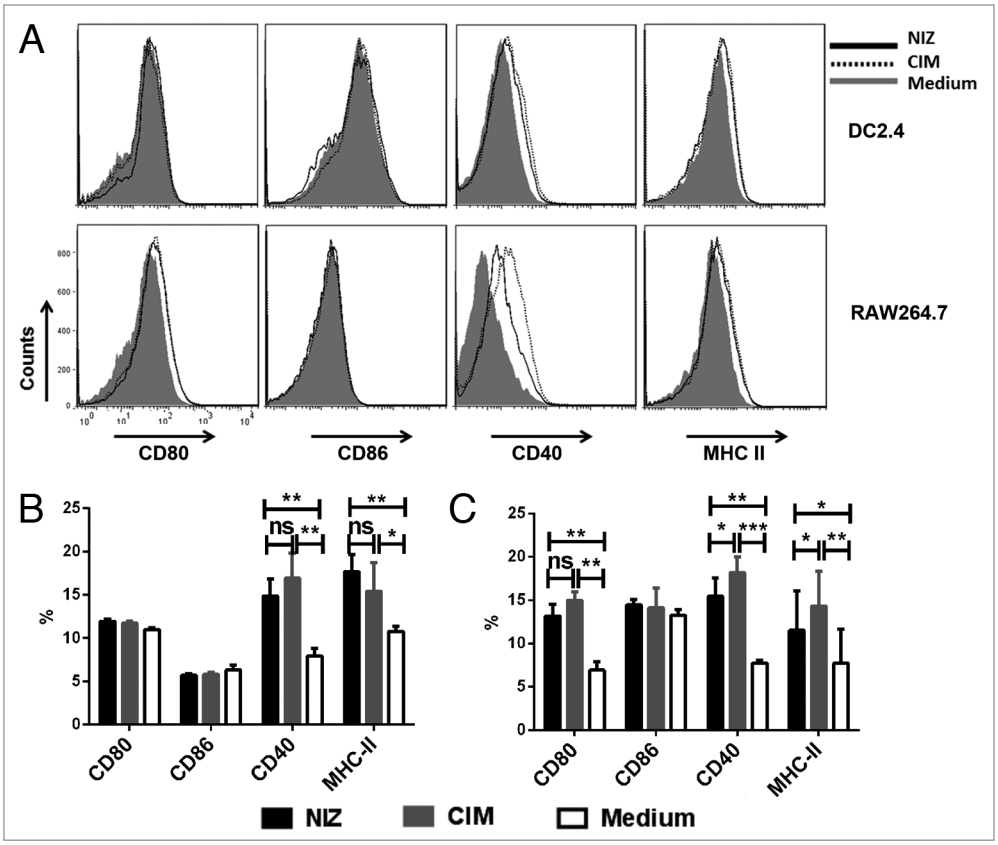

Figure 1. Effects of NIZ on APC activation in vitro. DC line DC2.4 and macrophage line RAW264.7 were treated with NIZ or CIM for 48 h. (**A**) The cells were immunostained for MHC-II, CD80, CD86 and CD40 and analyzed by FACS. (**B**) The percentage of cells bearing the markers in the DC2.4 cell line, summarized as the means of three independent experiments. (**C**) The percentage of cells bearing the markers in the RAW264.7 cell line summarized as means of three independent experiments. Cell fluorescence was gated for analysis and all data are presented as mean ± SD $^{*}P < 0.05$, $^{**}P < 0.01$ compared with medium treated cells.

and CIM compared with medium, and there were no significant differences between the treatments (**Fig. 1A and B**). MHC II, CD40, and CD80 were upregulated in RAW264.7 cell line after treatment with either NIZ or CIM, and the CIM treated group also showed a little higher expression of the MHC II and CD40, but there was no difference in CD80 level between the two groups (**Fig. 1A and C**). Only CD86 was not changed by either treatment in either cell line (**Fig. 1**). Thus overall the changes were similar after treatment with either agent.

Nizatidine enhances APC activation in vivo

To determine whether NIZ could promote the activation of DCs in vivo, we analyzed the expressions of CD80, CD86, and MHC II in the $CD11c^{+}$ cells (DCs) of spleens taken three days after the mice had been immunized with H5N1 killed viral antigen plus NIZ and CIM. The results showed that either NIZ plus antigen or CIM plus antigen gave enhanced expression of CD40, MHC II and CD86 compared with the H5N1 killed viral antigen alone. In the NIZ plus H5N1 antigen group, CD86 and MHCII expression levels were a little lower compared with those in the CIM plus vaccine group, but there was no difference in CD40 expression level (**Fig. 2A**). Overall the results suggested that NIZ could also stimulate DCs activation in vivo.

To determine in addition whether NIZ could enhance cytokine production, expression levels of IL-12, TNF-α, and IL-10 were examined by intracellular staining of splenocytes after immunization. Both NIZ and CIM could augment H5N1-induced upregulation of IL-12 and TNF-α, and suppress IL-10 expression in comparison to immunization with the H5N1 killed viral antigen alone. Expression of IL-12 was higher after NIZ plus antigen than after CIM plus antigen (**Fig. 2B**).

Nizatidine enhances humoral response

The level of anti-viral antibodies, particularly the property of hemagglutination inhibition, is a key standard used to evaluate the effectiveness of influenza vaccines. To test whether NIZ has the ability to enhance a humoral response to the H5N1 killed viral antigen, C57BL/6 mice were vaccinated once intramuscularly either with antigen alone or antigen plus NIZ. Antigen plus CIM was used as the positive control. On day 14 after the single immunization, antisera from the mice were analyzed by ELISA and assayed for hemagglutination inhibition (HI). Higher levels of total IgG and HI were elicited in mice vaccinated with H5N1 plus NIZ, compared with the control group immunized with H5N1 antigen alone (**Fig. 3A and B**), and HI titer was higher with NIZ than with CIM (**Fig. 3B**).

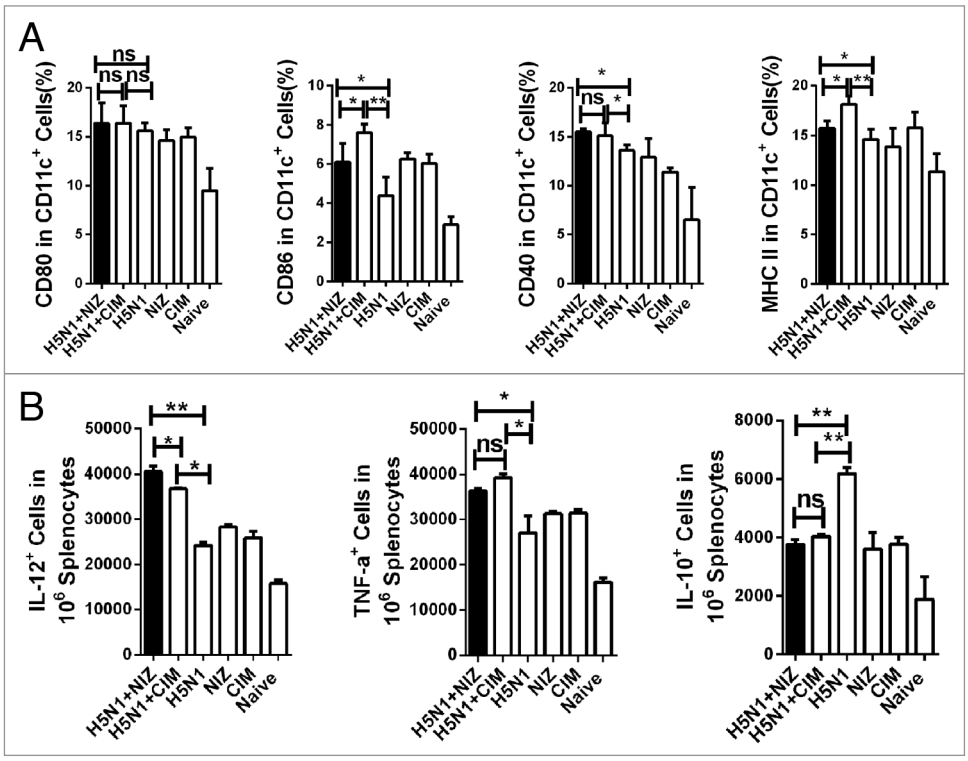

Figure 2. Analysis of splenocytes of C57BL/6 mice after immunization with H5N1 with or without NIZ. Samples were collected on day 3 after immunization and stimulated by 1 ug/ml H5N1 killed antigen for 12 h in vitro. (**A**) The CD11c+ cells were gated and analyzed for CD80, CD86, CD40, and MHC-II in total CD11c+ cells. (**B**) All the splenocytes were gated and intracellular immuno-staining for IL-12, TNF-α, or IL-10 analyzed by FACS. A representative result from three independent experiments is shown. All data are presented as mean ± SD *$P < 0.05$, **$P < 0.01$ compared with immunization with antigen alone.

The effects of Nizatidine as chemical adjuvant on CD4+ T-cell-mediated responses

Since viral specific CD4+ T-cell activity is indispensable for development of cytotoxic and antibody responses, we next tested whether NIZ could augment the cellular response to H5N1 killed viral antigen, again using CIM as a positive control. Functionality of antigen specific CD4+ T cells can be indicated by proliferative ability and elevated cytokine expression after re-encountering antigen. We examined T-cell proliferation and ability to express Th1 and Th2 cytokines on day 7 after a single vaccination and observed that H5N1 killed viral antigen plus NIZ or CIM could stimulate high levels of T-cell proliferation response, and there was no difference in effect between the two adjuvants. In contrast, antigen alone did not augment the T-cell response to any significant degree (**Fig. 4A**).

Frequencies of both IFN-γ and IL-4 cells in the antigen re-stimulated CD4+ T cell populations were increased significantly by both NIZ plus antigen and CIM plus antigen compared with antigen alone. The IL-4 level in the NIZ plus antigen group was a little higher than in the CIM plus antigen group (**Fig. 4B and C**). These findings suggest that NIZ is capable of promoting CD4+ T-cell responses in both proliferative and cytokine secreting aspects.

Nizatidine suppresses function of Treg cells

Another way to enhance immune responses is to inhibit a negative feedback loop, such as by inhibiting suppressor Treg cell function. To explore this possibility, the frequency of CD4+CD25+Foxp3+ Treg cells and their expression of IL-10 and TGF-β were examined by FACS and intracellular staining of the

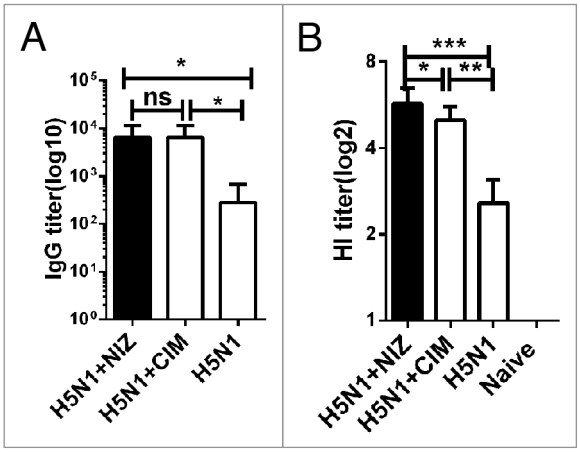

Figure 3. Effects of NIZ on IgG level. Serum samples were collected 14 d after a single immunization of C57BL/6 mice. Anti-H5N1 titer was determined by ELISA (**A**), or hemagglutination inhibition (**B**). Mean titers (n = 6) are expressed in log10 or log2. Representative results from three independent experiments are shown. All data are presented as mean ± SD *$P < 0.05$, **$P < 0.01$ compared with control mice vaccinated with killed viral antigen alone.

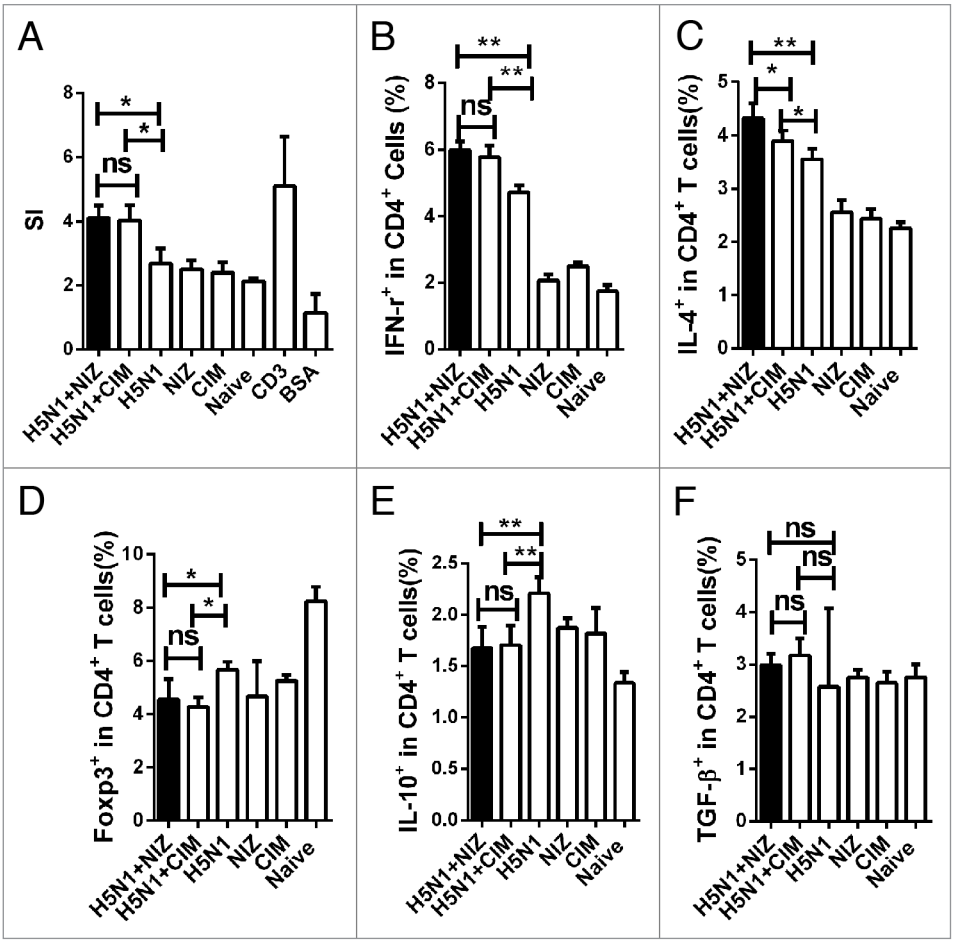

Figure 4. Effects of NIZ on T cell function. The splenocytes were separated 7 d after a single immunization and used to perform intracellular staining or for proliferation assay (n = 6). (**A**) The level of T-cell proliferation was assayed using MTT assay. The splenocytes were stimulated for 3 d in vitro using killed H5N1 antigen as a specific antigen, BSA as a non-specific antigen, or anti-CD3 as a positive control. Anti-CD28 was added as a co-stimulant in all reactions. Proliferative response was expressed as stimulation index (SI). Splenocytes were stimulated for 12 h in vitro using killed H5N1 antigen as a specific antigen and blocked with BFA for 8 h. The lymphocytes were first gated on $CD4^+$ T cells then stained for intracellular IFN-γ (**B**), IL-4 (**C**), Foxp3 (**D**), IL-10 (**E**) and TGF-β (**F**), and analyzed by FACS. Representative results from three independent experiments are shown. All data are presented as mean ± SD $^*P < 0.05$, $^{**}P < 0.01$ compared with mice vaccinated with killed viral antigen alone.

isolated splenocytes from each group of mice. The results showed that, compared with antigen alone, NIZ plus antigen or CIM plus antigen could not only reduce the proportion of CD4 cells that were differentiated as Treg cells (**Fig. 4D**), but could also downregulate the expression of IL-10. There was no difference between NIZ and CIM groups (**Fig. 4E and F**).

Nizatidine enhances protective response against H5N1 lethal challenge

To determine whether vaccination with the killed viral antigen plus NIZ could protect animals against lethal H5N1 viral challenge, mice were vaccinated with the antigen with or without NIZ or CIM and challenged on week two after the vaccination. The killed viral antigen alone slightly reduced the viral load seen in the lungs 6 d after infection. Significantly greater reduction of viral load was observed in the mice immunized with the antigen plus NIZ or CIM, and there was no difference between these two groups (**Fig. 5C**). Furthermore, the percentage of mice surviving 17 d after challenge was greater for mice vaccinated by the antigen plus NIZ (87%) than for those without adjuvant (14%) and those with CIM (57%)(**Fig. 5A**). The weight losses were also assessed during the course of the challenge infection. Immunization with the killed viral antigen alone or together with NIZ or CIM resulted in slower initial weight loss followed by weight gain (**Fig. 5B**). The controls lost weight faster and were all dead by day 11. Thus, these results provide evidence that nizatidine, an anti-H2 receptor antagonist that is more soluble than Cimetidine, can be readily used with the killed H5N1 viral formulation as a potent adjuvant.

Discussion

T cells have been proved to be essential for controlling influenza viral infections, yet the widely used formalin-inactivated vaccines do not induce robust T-cell responses specific for influenza virus antigens.[8,22,23] Antibody to neutralize viral entry is the key factor for successful development of prophylactic flu vaccines. Recent

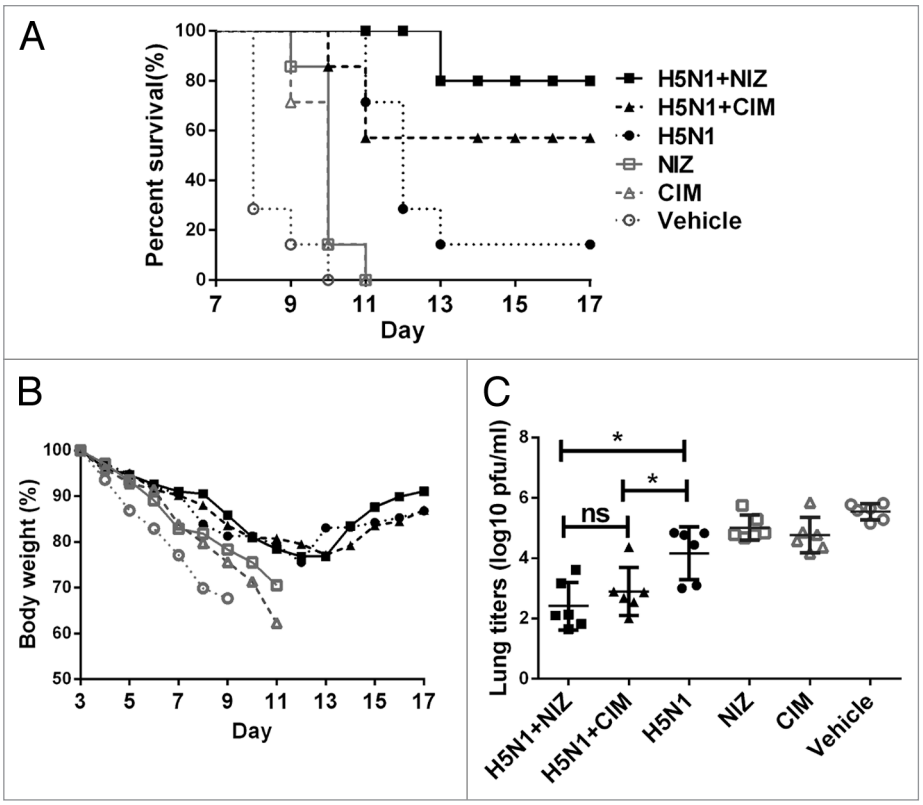

Figure 5. Effects of NIZ on the protective responses. Seven mice in each group were immunized once with the killed H5N1 antigen, with or without NIZ, 14 d before challenge with 10 LD_{50} per animal. (**A**) Survival curves after H5N1 challenge were recorded. (**B**) Body weight change of challenged mice was measured every day and (**C**) viral loads in the lungs of each animal were measured 7 d after the challenge. All data are presented as mean ± SD *$P < 0.05$, **$P < 0.01$ compared with mice immunized with the killed H5N1 antigen alone.

studies showed that modulations of antibody and $CD8^+$ T-cell responses by $CD4^+$ T helper (Th) cells are essential. Balanced immune responses could be achieved only by a proper stimulation for a certain differentiation and biased response of Th cells.[8,24] Dysregulated Th cell function often leads to inefficient clearance of pathogens.[25] When properly regulated, Th1 cells help the cell mediated response that includes CTL functions against infected cells and Th2 cells help B cells to produce antibody response to prevent virus entry into cells. These responses could be influenced by the addition of adjuvants that act at the early stage of DC activation and maturation. The DCs are involved in T-cell activation through antigen presentation in the context of MHC molecules and co-stimulatory molecules on the cell surface. It has been demonstrated that the expression of MHC molecules and the expression of co-stimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) can modulate T-cell activation and Th1/Th2 polarization during infection and autoimmunity.[26,27] CD40 is a co-stimulatory molecule found to be essential in mediating a broad variety of immune and inflammatory responses including T-cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.[28-30] In this study, we found that NIZ was able to enhance T helper cell responses to H5N1 killed viral antigen and improve protection against H5N1 virus. We demonstrated that NIZ could (1) activate APC by upregulating co-stimulation markers, (2) augment $CD4^+$ T-cell immune responses to the H5N1 killed viral antigen that lead to enhanced antibody HI titer and CTL responses, and (3) suppress the function of Treg cells, especially downregulating the IL-10 expression.

CIM, a similar H2-receptor antagonist, has been demonstrated to induce high levels of T cell and antibody responses when mixed with a DNA vaccine encoding the HBV surface antigen.[20,21] Although here both NIZ and CIM at the same molar concentration induced similar immune responses to the killed H5N1 viral antigen, NIZ promoted higher levels of IL-12, IL-4 and HI titers, and achieved a higher level of protection (85% vs. 57%) against a lethal dose of H5N1 virus challenge (**Fig. 5A**). The differences may be due to the different molecular compounds interacting with different immune pathways even though they both interact with histamine H2 receptor and have similar anti-stomach acid physiological function. These results indicate that NIZ is a more efficient adjuvant for H5N1 vaccine.

The adjuvant could not only activate the $CD4^+$ T-cell functions, but more importantly could also inhibit the suppressive function of Treg cells, by downregulating the Foxp3 and IL-10 expression (**Fig. 2B–E**). Recent research indicates that Foxp3 downregulation can also be induced in nTreg in vitro and in vivo after stimulation in the presence of pro-inflammatory signals or in the presence of exogenous-activated DC.[31] DC develop via immature DC (imDC), that show low antigen

presenting capacity with little expression of co-stimulatory and MHC II molecules, into mature DC (mDC), that possess the strongest antigen processing and presenting ability with high expression levels of co-stimulatory and MHC II molecules on the cell surface.[32] imDCs could significantly induce Treg differentiation, whereas mDCs isolated from immunized mice suppressed Treg differentiation in vitro, suggesting that potent immunization stimulates DC maturation and thereby dampens Treg differentiation.[33] In our data (**Figs. 1 and 2**), we found that the mDC marker MHC II and co-stimulatory molecules were upregulated in all the immunization groups, but reached the highest levels in the H5N1+NIZ/CIM group. This may explain why the adjuvant used in our study could inhibit the Treg suppressive function. In addition, our data showed that the adjuvant affects the DCs from day 2 in vitro and day 3 in vivo (**Fig. 1 and 2**), but no difference between immunized and naive groups before day 1 (**Fig S2**). Furthermore, IL-10 is an immunoregulatory cytokine that downregulates the functions of antigen-presenting cells, T-cell activation, and the production of proinflammatory cytokines.[34] Many studies have shown that blocking IL-10 signaling in virus-infected mice enhanced $CD4^+$ T cell production of IFN-γ and increased serum anti-viral IgG1 levels[35]; for example, monoclonal antibody-induced blockade of IL-10 receptor (IL-10R) generated a favorable balance of $CD4^+$ T-cell responses to virus.[36] Consistent with this, our results (**Figs. 2B and 4E**) showed that the level of IL-10 expression was significantly downregulated in the H5N1+NIZ/CIM treated group, so this may be another mechanism underlying the enhanced immune responses observed during H5N1+CIM/NIZ priming.

Two obvious advantages for NIZ as a vaccine adjuvant over CIM are (1) its high solubility in water, which makes it easier to formulate with antigens, including as in this case, killed virus; (2) its lower level of toxicity.[15,37,38] These two practical advantages and its potency in protection could move Nizatidine into clinical trial for use as a vaccine adjuvant. Notably, Nizatidine may represent a promising chemical adjuvant for killed vaccine development when both humoral and cellular responses are required.

Materials and Methods

Animal

Adult female C57BL/6 mice at 8–10 wk of age were purchased from Animal Institute of Chinese Medical Academy. The protocol of all animals used in the experiments were approved by the Animal Welfare Committee of China Agricultural University and housed with pathogen-free food and water under 12 h light-cycle conditions.

Vaccines

Whole H5N1 (Re-6) chemically inactivated viruses at 100 EID50/ml (50% egg infectious dose) was used by treating with 0.2% formalin at 37 °C for 24 h have killed viral antigen (provided by Dahuanong Animal Health Inc.). The killed viral antigen was then tested for its immunogenicity in mice and inability to infect chicken embryos. The protein concentration of the antigen was determined with Pierce BCA Protein Assay Kit (Pierce, cat. no. 23227).

Immunization

Nizatidine (NCPC, cat. no. 76963-41-2) was dissolved in PBS to 20 mg/ml. Cimetidine (Sigma, cat. no. C4522) was initially dissolved in 0.15 M hydrochloric acid with saline and then adjusted to neutral pH with drops of 1M sodium hydroxide and subsequently diluted to 1% with the saline solution. The C57BL/6 mice were randomly divided into five groups (n = 10 each). The 100 ul of 0.5 ug killed viral antigen were formulated with or without adjuvant (NIZ: 0.5 ug antigen with 0.62 mg NIZ/mice; CIM: 0.5 ug antigen with 0.5% of CIM/mice), injected intramuscularly once.

Virus challenge

Two weeks after the single dose of immunization, mice were challenged with a 10 lethal dose (LD_{50}) of mouse-adapted strain A/Chicken/Henan/1/04 (H5N1) by intranasal administration of 20 ul of the viral suspension. Following infection, three mice were sacrificed to remove their lungs on day 6 for analyzing lung viral loads whereas the remaining 7 mice were monitored daily for morbidity assessed by measuring body weight loss and survival for up to 17 d. Individual body weights were recorded for each mouse on various days post-infection. This study has been repeated for additional two times.

Detection of anti-H5N1 antibody

Serum anti-H5N1 antibody was measured with an enzyme linked immunosorbent assay (ELISA). On day 14 after immunization, serum was collected. ELISA was performed in a 96-well polystyrene microliter plate (Corning, cat. no. 3590) with reagents consisting of H5N1 killed viral antigen. Following the blockage with 3% of BSA (Sigma, cat. no. A1933) for 1 h, the plate was incubated with diluted mouse sera in each well. A second goat anti-mouse IgG Ab (Santa, cat. no. sc-2005) diluted 1000 times was added. Ten milligrams of TMB tablet (Sigma, cat. no. T2885) was dissolved by 0.025 M phosphate-citrate buffer and subsequently added to each well for color development. After the reaction was stopped by 2 M H_2SO4, the plate was read with a plate reader (Magellan) at 450 nm. The antibody titers were determined by the absolute ratio of OD values of post/naive sera at a cutoff ratio of 2:1. Sera were pooled from groups of 6 mice, and this study was repeated an additional two times.

Hemagglutination inhibition (HI) test

Serum was collected on day 14 after single immunization. The serum samples were diluted at 2-fold dilutions until at 1:1024 made by saline, and incubated with 25 ul of 1:512 of diluted H5N1 virus in a U-bottom microtiter plates (Corning, cat. no. 3799) for 30 min, followed by incubation with 25 ul of 1% chicken red blood cells for 30 min. The results were determined by the reciprocal of the highest dilution of sera samples that completely inhibits hemagglutination. Every group contained 6 mice, and this study was repeated an additional two times.

Treatment of DC and macrophage cell lines

DC2.4 and RAW264.7 cells were plated at 5×10^6/ml in six-well plates (Corning, cat. no. 3516). NIZ was added at

6 ug/ml, and CIM was added at 5 ug/ml. After 48 h, the cells were surface stained by APC-anti-CD80 (ebioscience, cat. no. 17-0801), PE-anti-CD86 (ebioscience, cat. no. 12-0862), PE-anti-CD40 (ebioscience, cat. no. 12-0401) and APC-anti-MHC II (ebioscience, cat. no. 17-5321) in PBS and 1% BSA (Sigma, cat. no. A1933) for 30 min at 4 °C, and analyzed by FACS Aria I (BD Biosciences).

T-cell proliferation

T-cell proliferation was evaluated by the MTT colorimetric assay.[39] Splenocytoes were separated 7 d after single immunization and cultured in triplicates using 96-well round-bottom plates at 5×10^5 cells/ml in RPMI 1640 medium (GIBCO, cat. no. 11875093) containing 5% FCS (GIBCO, cat. no. 10099141) at 37 °C in a 5% CO_2 incubator, and re-stimulated with the killed virus H5N1 antigen, anti-CD3 (AbD Serotec, cat. no. MCA500G) and anti-CD28 (eBiosciences, cat. no. 16-0281) (positive Control), BSA (Sigma, cat. no. A1933) or no antigen (negative control). T-cell proliferation was evaluated by MTT colorimetric assay. The OD values of plates were read at 570 nm by a plate reader (Magellan). Data were expressed as stimulated index, calculated as the mean reading of triplicate wells stimulated with an antigen, divided by the mean reading of triplicate wells stimulated with medium. Every group contained 6 mice, and this study was repeated an additional two times.

Flow cytometric analysis

Single T-cell suspensions were prepared 7 d after single immunization and stimulated with 1 ug/ml H5N1 killed viral antigen in the presence of BFA (5 ug/ml, BD Biosciences, cat. no. 555029) for 10 h at 37 °C and 5% CO_2. Collected cells were stained with appropriate conjugated FITC-CD4 (eBiosciences, cat. no. 11-0041) in PBS and 1% BSA (Sigma, cat. no. A1933) for 30 min at 4 °C, and fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. For intracellular staining we stained the antibody of PE-anti-IL-4 (ebioscience, cat. no. 12-7041), PE-anti-IFN-γ (ebioscience, cat. no. 12-7311), APC-anti-IL-10 (ebioscience, cat. no. 17-7101), PE-anti-TNF-α (ebioscience, cat. no. 17-7321), PE-anti-IL-12 (ebioscience, cat. no. 17-7123), PE-anti-TGF-β (Biolegend, cat.no.141306) and APC-anti-Foxp3 (eBiosciences, cat. no. 17-5773). Samples were analyzed by FACSAria I (BD Biosciences). Every group contained 6 mice, and this study was repeated an additional two times.

Viral RNA determination

Total RNA was prepared from 10 mg lung homogenized and extracted in Trizol (Invitrogen, cat. no. 15596018) according to the manufacture's instruction. DNase I (Takara Inc., cat. no. 2270A) treated RNA (2 mg) was used to reverse transcribe into cDNA using a set of universal primers for influenza A virus[40] as following: forward primer, 5′-CGCAGTATTC AGAAGAAGCA AGAC-3′; reverse primer, 5′-TCCATAAGGAT AGACCAGCTACC A-3′. Real-time PCR was performed to amplify the hemagglutinin (HA) gene of H5N1 influenza virus using SYBRH PrimeScriptH RT-PCR Kit (TaKaRa Inc. cat. no. DRR066A). The reaction was run on an ABI 7500 and data analysis was performed using 7500 software v2.0 (ABI). The copy number of the HA gene was calculated by using a HA-containing plasmid of known concentration as a standard.

Statistical Analysis

Results are presented as means ± SD. One-Way ANOVA was used for data analysis, two groups compared were used paired-T test to analysis. A value of $P < 0.05$ was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/vaccines/article/27165

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