

An MF59-Based Nanoemulsion Adjuvant Couples Immunogenic Cell Death with Trained Immunity for Potent RSV Vaccine Responses

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Abstract

Respiratory syncytial virus (RSV) poses a serious threat to the health of infants, young children, and the elderly. The development of RSV vaccines faces challenges such as Th2-biased immunopathology and insufficient immune persistence. The MF59 adjuvant has been safely used in influenza vaccines for 30 years. In this study, based on MF59, we constructed a novel composite nanoemulsion adjuvant, MF59@MDP, by incorporating the immunostimulator MDP. We exploited damage-associated molecular patterns (DAMPs), such as ATP and dsDNA, released upon tissue injury induced by high local concentrations of Span85 and Tween80 at the injection site, to promote antigen-presenting cell-mediated trafficking of MDP to lymph nodes, enhance pro-inflammatory cytokine release from lymph node dendritic cells (DCs), and modulate their epigenetic modifications. Through reprogramming of innate immune cells, this strategy corrected T cell immune bias and synergistically enhanced both humoral and cellular immunity. Furthermore, combining MF59@MDP with CpG further augmented its trained immunity potential. Mouse challenge experiments demonstrated that the MDP-containing adjuvant group more readily induced cytotoxic T lymphocyte (CTL) responses and effectively protected against viral challenge. This thesis aims to construct a novel MF59-based composite nanoemulsion adjuvant to promote the generation, persistence, and immune memory formation of RSV preF antigen-specific humoral and cellular immunity, thereby laying a foundation for the development of novel recombinant RSV vaccines.

Introduction

Respiratory syncytial virus (RSV) is a negative-strand RNA virus belonging to the family Paramyxoviridae^[1]. It has been found that most infants and young children are infected with RSV, and even multiple infections occur. Infants and young children are caused by acute lower respiratory tract infections after virus invasion, resulting in an increase in infant mortality^[2]. A survey in 2015 showed that approximately 55,000 to 19,900 child deaths each year worldwide were caused by acute lower respiratory tract infections directly or caused by respiratory syncytial virus^[3]. In addition, respiratory syncytial virus can also infect the elderly or young people with low immunity^[4]. The elderly, as a population with low immunity, face more risks when they are infected with high-risk viruses^[5].

The whole genome of respiratory syncytial virus is 15.2kb, which contains ten genes encoding structural and non-structural proteins. Among them, ribosomal proteins include^[6]: Nucleoprotein (N), Phosphoprotein (P), Large polymerase protein (L), Matrix 2 proteins (M2) (including M2-1 anti-termination protein and M2-2 protein)^[7]; nonstructural proteins NS (Nonstructural Proteins, NS)^[8] include: NS-1 and NS-2^[9]; envelope proteins include: Matrix proteins (M)(binding to intracellular F protein), Small hydrophobic protein (SH)^[10], Attachment glycoprotein (G)^[11], Fusion protein (F)^[12]. Among them, SH protein is a pentameric ion channel. Studies have shown that small hydrophobic proteins are involved in delaying the apoptosis of infected cells, and that SH deficiency does not affect the membrane fusion of respiratory syncytial virus^[10]. G protein and F protein are the two most

important transmembrane proteins of respiratory syncytial virus, which play an important role in the entry of the virus into the cell^[13]. Among them, G protein mainly mediates the adsorption of the virus to the cell membrane^[14], while F protein mainly mediates the fusion of the virus and the cell membrane^[15].

In the 1860s, Pfizer developed the formalin-inactivated vaccine (FI-RSV) vaccine and conducted clinical trials in infants. However, the inactivated vaccine not only failed to protect serum-negative infants from RSV infection^[16], but also led to severe enhanced respiratory disease (ERD)^[17], 80% of infants were severely hospitalized after RSV infection, and 2 of them died. Subsequent studies have shown that the F protein conformation of the inactivated RSV mainly contains a post-fusion conformation (post-Fusion)^[18], rather than a pre-fusion conformation (pre-Fusion), and the fused F protein lacks an antigen conformational epitope that induces neutralizing antibodies^[19]. Therefore, FI-RSV leads to the destruction of neutralizing epitopes and the lack of sufficient neutralizing antibodies is one of the causes of ERD. In addition, Th2-biased cell response induced by FI-RSV is another possible cause of ERD^[20].

As of July 2023, only two bivalent vaccines, Arexvy of GSK's DS-Cav1 mutant^[21] and Abrysvo of Pfizer's 847 mutant, have been approved in the United States^[21], but they are also only used to prevent lower respiratory diseases caused by RSV infection in people over 60 years old. Unfortunately, at present, there is no RSV vaccine approved for the prevention of RSV infection in China, which is in the stage of preclinical research or clinical trial.

Adjuvants are also needed to be added to the virus prevention immunity of the elderly to achieve sufficient prevention of virus invasion^[22]. Infants are mainly immunized with antibodies against respiratory syncytial virus F protein. However, due to the short window of infection, the rapid production of neutralizing antibodies against the virus after vaccination is the key to the success of the vaccine^[23]. In addition, because people will be repeatedly infected with respiratory syncytial virus in their lifetime, the establishment of long-term immune memory is an important direction for infants and the elderly in vaccine prevention and treatment^[24]. The immunogenicity of subunit vaccine is poor. Therefore, first, the design concept of good antigen is the key to the success of respiratory syncytial virus. Second, Th2-biased effects such as aluminum adjuvants also need to be improved^[25], and Th1-biased adjuvants can improve the immune effect of vaccines^[26, 27].

At present, the mature adjuvants are Aluminum-containing adjuvants (Alum), Oil-in-Water Emulsion Adjuvant (MF59), Freund's adjuvant (CFA), Adjuvant System (AS) and so on. GSK uses the AS01 in the AS series developed by itself as an important adjuvant part. Studies have shown that the lack of T cell responses such as CD4 to DS-Cav1 protein combined with adjuvants may be the cause of the generally low-quality humoral responses to protein-based vaccines observed in mice^[28]. Respiratory syncytial virus infection in infants and young children death is due to the newborn's trachea is thin, after infection due to shedding of epithelial, mucus, fibrin and inflammatory debris accumulation lead to small airway obstruction^[29]. At present, the solution for infants infected with respiratory syncytial virus is to improve the immunity of newborns to respiratory syncytial virus by increasing the titer of maternal

immunization^[30]. Therefore, the development of an adjuvant that can enhance humoral and cellular immunity is one of the key factors in the successful development of respiratory syncytial virus vaccines^[31].

MF59 is a clinically available safe adjuvant, which is composed of squalene, polysorbate 80 (Tween 80), sorbitol anhydride oleic acid ester (Span 85) and citric acid buffer^[32]. The MF59 adjuvant flu vaccine (Flaud) has now been licensed in more than 20 countries around the world, with more than 85 million doses administered^[33]. MF59 adjuvants recruit immune cells by creating an 'immune microenvironment' at the injection site. These immune cells phagocytize antigens and adjuvants and transport them to local lymph nodes.

Since MF59 is a Th2-biased adjuvant and lacks cellular immunity, a series of studies have given MF59 other immune mechanisms by adding immunostimulants and engineering modifications. First, MF59 combined with other receptor stimulators^[34]. In mice, the addition of CpG ODN adjuvant (CpG oligodeoxynucleotides, CpG) after MF59 combined with HSP65-MUC1 found that it gave Th1 immune bias and played a significant role in the treatment of melanoma in mice^[35]. Second, the squalene core is supplemented with other immunomodulators to regulate the immune response such as: AS01, MF59. Due to the lack of cellular immune stimulation, in the study of influenza A H5N1 virus (H5N1) vaccine, it was found that AS03 statistically significantly caused a wider heterotypic antibody response. The AS03 adjuvant group showed an increase in the specific binding percentage of its HA and HB antigens to IgG compared to the MF59 group after the second dose, while the MF59 group showed a decrease^[36]. Third, by replacing the surfactant that forms oil-in-water, AF03 forms a new o / w nanoemulsion adjuvant that changes particle size and immune function by replacing the surfactant polyoxyethylene cetyl ether, mannitol, and dehydrated sorbitol monooleate^[37]. By replacing the surfactants with sorbitol trioleate and polyoxyethylene sorbitol monooleate, the Sepivac SWE TM can greatly enhance the humoral and cellular immune responses of the SARS-CoV-2 receptor binding domain (RBD) dimer immunogen^[38].

MF59 is not only an adjuvant with high biosafety, but also a good nano-delivery system. The addition of a cationic surfactant (1,2-Dioleoyl-3-trimethylammonium-propane, DOTAP) to the formula of MF59 makes the surface of MF59 positively charged, making MF59 have the potential to deliver mRNA^[39].

The most important research obstacle of respiratory syncytial virus is the immune bias caused by vaccine immunization. In order to enhance the adaptive immunity after immunization and the secretion of Th1-biased cytokines such as IFN- γ and IL-2, increasing Th1-biased inducers is the focus of vaccine development^[40]. Adjuvants are often added to vaccines to enhance the intensity, breadth and persistence of the immune response. They are mainly used in traditional vaccines such as inactivated vaccines, protein or peptide vaccines. We speculate that the tendency of Th2 immune bias induced by mutant protein vaccines is related to the addition of aluminum adjuvants. Traditional aluminum adjuvants usually only induce Th2-type responses. For diseases caused by viral infections, it is

necessary to directly kill infected and diseased cells through the function of Th1-activated macrophages, B cells and CD8⁺ cytotoxic T cells^[41]. Therefore, we can choose adjuvants that change Th immune bias.

Innate immune cells are crucial in the development of anti-tumor immune surveillance and anti-tumor adaptive cellular immunity^[42, 43]. Well-trained innate immune cells exhibit characteristics similar to immune memory, and produce stronger immune responses to secondary homologous or heterologous stimuli^[44]. When pattern recognition receptors (PRRs) receive pathogens or endogenous danger signals, the innate immune system is activated to stimulate the activation of adaptive immunity and establish immune memory against pathogens or danger signals^[45, 46]. Nucleotide binding oligomerization domain containing 2 Gene (NOD2) is an intracellular signaling receptor. Cells are stimulated by Muramyl Dipeptide (MDP). NOD2 oligomerizes and binds to receptor interacting protein kinase 2 (RIPK2)^[47], which subsequently leads to the activation of classical NF-κB and MAPK pathways^[48].

Poly (lactic-co-glycolic acid) (PLGA) was used to deliver MDP. The results showed that innate immune cells such as dendritic cells (DC), macrophages (MØ) and monocytes established immune memory against pathogens. When the body is subsequently inoculated with pathogens, the body quickly establishes an immune response, which ultimately inhibits or even eliminates tumor growth^[49]. MDP can not only train the body's innate immune mechanism, but also stimulate the body's humoral immunity^[50].

Successful vaccine design for respiratory syncytial virus requires a biologically good safety adjuvant that significantly enhances humoral and cellular immunity.

MF59 is a good bio-safe adjuvant, but due to its own functional defects, it cannot trigger the corresponding activation of cellular immunity. MDP, as a training immune stimulator, has the function of training innate immune cells. Therefore, combined with MF59, MDP has the function of recruiting inflammatory cells, especially monocytes, macrophages, DC cells and other cells at the injection site and being engulfed by cells with strong phagocytic ability, so that MDP has the ability to target innate immune cells and be recognized by intracellular NOD2 recognition receptors to activate NF-κB and MAPK signaling pathways. The new training immune nanoemulsion adjuvant MM59 prepared based on the idea of training immunity will have the function of enhancing humoral immunity of MF59, and give the nanoemulsion adjuvant the function of training immunity mechanism and triggering adaptive immunity.

Results

2.1 The morphology and physical properties of MF59@MDP and the evaluation of inducing BMDC to establish training immunity.

MF59 is a well-established clinical adjuvant composed of safe components and associated with no apparent adverse effects. Although it effectively enhances antibody production, its inability to induce cellular immunity makes it unsuitable for respiratory syncytial virus (RSV) vaccine design. To overcome

this limitation, we introduced muramyl dipeptide (MDP) to modulate post-immunization immune bias through the mechanism of trained immunity.

We first optimized the preparation procedure for the MF59 oil-in-water nanoemulsion. Transmission electron microscopy (TEM) revealed that the optimized MF59 adjuvant formed uniform nanoparticles (Fig. 1A). We compared the IgG antibody levels induced by our in-house MF59 oil-in-water nanoemulsion combined with 10 µg of RSV preF antigen DS-Cav1 with those induced by DS-Cav1 without adjuvant or by antigen combined with the foreign reference adjuvant AddaVax or the domestic reference adjuvant BAF03. With or without CpG, the in-house MF59 exhibited antibody-inducing capacity comparable to that of AddaVax and BAF03 (Supplementary Fig. 1A&B). Following the same procedure, MF59@MDP prepared by incorporating MDP into the oil phase also exhibited homogeneous nanoparticles (Fig. 1B). After high-pressure homogenization, MF59 yielded nanoparticles with a polydispersity index (PDI) of 0.07 and an average diameter of 171.2 nm (Fig. 1C). After storage in vials at 4°C, 37°C, and 50°C for 30 days, MF59 showed the smallest change in particle size at 37°C (Fig. 1D), the lowest variation in zeta potential (Fig. 1E), and no significant alteration in PDI (Fig. 1E). For MF59@MDP, after MDP incorporation and the same homogenization process, the resulting nanoparticles had a PDI of 0.09 and a mean size of 184.9 nm (Fig. 2G). After storage under the same temperature conditions for 30 days, the particle size of MF59@MDP remained almost unchanged at both 37°C and 50°C (Fig. 1H), zeta potential showed no obvious variation (Fig. 1I), and PDI remained lower at 37°C and 50°C (Fig. 1J).

We further compared three Nile Red-labeled formulations: PBS and MF59@MDP with or without high-pressure homogenization—in terms of particle size and PDI (Fig. 1K). After homogenization, MF59@MDP exhibited a significantly reduced particle size compared with its non-homogenized counterpart and PBS (Fig. 1L), with the average PDI decreasing to 0.04 (Fig. 1M). When bone marrow-derived dendritic cells (BMDCs) were incubated with Nile Red-labeled MF59@MDP and extensively washed to remove unbound dye, confocal microscopy clearly demonstrated that BMDCs could internalize the nanoparticles. This observation provides a prerequisite for the intracellular delivery of MDP, enabling its action within antigen-presenting cells (Fig. 1N).

In addition, BMDCs were incubated with PBS, MF59, MDP, or MF59@MDP for 2 h, after which cell lysates were collected for Western blot analysis to evaluate the activation of signaling pathways associated with trained immunity (Fig. 1O). Both MF59@MDP and free MDP induced AKT phosphorylation; notably, the nanoformulated MF59@MDP elicited a stronger response than the small-molecule MDP (Fig. 1P).

2.2 High concentration of MF59@MDP induces cell death and releases immunogenic substances, enhancing DC maturation and antigen presentation.

Previous studies have shown that the MF59 nanoemulsion adjuvant can insert its surfactant components into the cell membrane within minutes after injection, thereby altering membrane fluidity and inducing transient membrane permeabilization. This leads to the release of large amounts of immunogenic substances, including ATP and lactate dehydrogenase (LDH). ATP, acting as a "find-me" signal, plays a crucial role in recruiting antigen-presenting cells (APCs).

First, we observed that this physical stimulation induced cell death in a dose-dependent manner: as the volume of adjuvant per 100 μ L of culture medium increased, the extent of cell death became more pronounced (Fig. 2A). Notably, no cell death was observed at low doses; however, once a threshold dose was exceeded, the effect became apparent and progressively intensified with increasing doses. This pattern of cell death correlated well with the release of the immunogenic molecule dsDNA (Supplement Fig. 2A&B). We then stimulated 293T cells with MF59@MDP at an adjuvant-to-medium volume ratio of 4:1. After 24 h of incubation, evident cell death was observed (Fig. 2B). In the culture supernatants, the cell death marker LDH was significantly elevated in the MF59 and MF59@MDP groups compared with controls (Fig. 2C). The release of the "Find-me" signal ATP was substantially higher in these groups than in the PBS and free MDP groups (Fig. 2D), and a marked release of dsDNA was also detected (Fig. 2E).

Subsequently, we collected the supernatants from these stimulated cells and incubated them with bone marrow-derived dendritic cells (BMDCs) overnight to assess whether the released immunogenic substances and "find-me" signals could promote BMDC maturation and enhance antigen-presenting molecule expression. Flow cytometric analysis revealed that the supernatant from the MF59@MDP group significantly upregulated the maturation markers CD80 and CD86 (Fig. 2F&G) as well as the antigen-presenting molecules MHC-I and MHC-II (Fig. 2H&I) on BMDCs.

Taken together, these findings indicate that both MF59 and MF59@MDP can induce immunogenic cell death (ICD) at the injection site immediately after administration, owing to the relatively high local adjuvant concentration. However, as adjuvant molecules are progressively internalized by cells and transported to draining lymph nodes, the local density decreases, thereby attenuating the ICD effect. Nonetheless, the "find-me" signals and immunogenic substances generated during ICD exert a potent effect on recruiting APCs and promoting their maturation. Therefore, the ICD effect elicited by MF59@MDP at high doses provides a prerequisite for achieving lymph-node-targeted delivery and training immunity in antigen-presenting cells.

2.3 The ICD promotes the enrichment of APC at the injection site so that the APC carry MF59@MDP for lymph node homing.

MF59@MDP, via the physical stimulation exerted by surfactants upon high-concentration aggregation, induces immunogenic cell death (ICD) in cells at the injection site. Immunogenic substances released during ICD, such as ATP and dsDNA, not only induce antigen-presenting cell (APC) maturation but also activate tissue-resident cells to release abundant chemokines, further recruiting additional immune cells and forming a positive feedback loop. dsDNA is a potent cGAS-STING agonist and can act on tissue-resident APCs to promote the release of large amounts of proinflammatory cytokines and chemokines. ATP acts on receptors on the surface of dendritic cells (DCs), inducing the release of abundant chemokines. These danger signals facilitate APC recruitment to the injection site and promote nanoparticle phagocytosis, thereby enabling nanoparticle accumulation in lymph nodes.

Nile Red-containing PBS, -MF59@MDP, and +MF59@MDP were injected into the left thigh muscle of mice. The results showed that nanoformulated MF59@MDP tended to exhibit higher fluorescence intensity in muscle (Fig. 3A), and +MF59@MDP accumulated in the draining lymph nodes ipsilateral to the injected muscle (Fig. 3B). Lymph nodes were collected from mice injected with Nile Red-labeled MF59@MDP into the left muscle; MF59@MDP was mainly present in the draining lymph nodes on the injection side (Fig. 3C). Subsequently, the time-dependent decay of Nile Red-labeled MF59@MDP in mouse muscle tissue and the kinetics of its accumulation in draining lymph nodes were examined. In vivo imaging showed that Nile Red fluorescence gradually decreased over time after injection (Fig. 3D). At 72 h after MF59@MDP injection, the fluorescence intensity had decayed to a level comparable to that of the PBS group (Fig. 3E). qPCR was used to examine the time-dependent upregulation of chemokine expression in muscle. The results showed that MF59@MDP markedly upregulated CCL2, CCL3, and CCL4 after injection (Fig. 3F–H); this trend was consistent with the fluorescence intensity profile of MF59@MDP, whereas CCL5 was only weakly upregulated, indicating that MF59@MDP induces CCL5 to a relatively low extent (Fig. 3I). This suggests that MF59@MDP is more effective in recruiting APCs such as monocytes and macrophages, but has a weaker capacity to recruit T cells. Over time, increasing amounts of MF59@MDP migrated from the injection site to the draining lymph nodes to exert adjuvant effects, with this phenomenon peaking at 48 h (Fig. 3J). We found that CD11c⁺ DCs (Fig. 3K) and CD11c⁺ F4/80⁺ macrophages (Fig. 3L) in the draining lymph nodes were increasingly recruited over time, indicating that this adjuvant promotes APC accumulation in the draining lymph nodes. In addition, flow cytometric analysis showed that Nile Red⁺ cells exhibited a time-dependent decrease similar to that observed by in vivo imaging (Fig. 3M). The signals of Nile Red-phagocytosing DCs and macrophages showed an initial increase followed by a decrease over time, indicating that MF59@MDP was gradually internalized and degraded within cells (Fig. 3N&O). These findings indicate that MF59@MDP has the potential to achieve lymph node accumulation and to utilize MDP to establish trained innate immunity in innate immune cells within lymph nodes.

2.4 The training immune mechanism and adjuvant effect of MF59@MDP in draining lymph nodes

In the preceding sections, we established that these nanoparticles are highly enriched in lymph nodes and that Nile Red dye is also present in innate immune cells such as DCs and macrophages. Therefore, DCs in the lymph nodes have the potential to undergo trained immunity induction. Mechanistically, MDP establishes trained immunity by entering cells via endocytosis and binding to the intracellular NOD2 receptor, thereby inducing mTOR phosphorylation. Phosphorylated mTOR further promotes downstream AKT phosphorylation and induces inflammatory cytokine expression. In isolated BMDCs, MF59@MDP exhibited the same capacity as the small-molecule MDP to induce production of the inflammatory cytokines IL-6 and TNF- α (Fig. 4A&B). Subsequently, these nano-adjuvants were injected into the hind leg muscle of mice. Three days later, draining lymph nodes (dLNs) were isolated, and flow cytometric analysis of cell phenotypes in the dLNs showed significant increases in the DC maturation marker CD80 and the antigen-presenting molecule MHC-I (Fig. 4C&D). Isolated lymphocytes were then subjected to secondary heterologous stimulation with 100 ng/mL LPS *in vitro*. Lymph node cells from the MF59@MDP group exhibited an enhanced capacity to produce IL-6 and TNF- α after training (Fig. 4E&F). Immunofluorescence sections of the dLNs revealed that the adjuvants enhanced the enrichment of CD11c⁺ cells in the lymph nodes, with the most pronounced effect observed in the MF59@MDP group (Fig. 4G). We then immunized BALB/c mice with 25 μ L of PBS, MF59, MDP, or MF59@MDP, each combined with 5 μ g RSV preF antigen vaccine. Through the synergistic action of MF59 and MDP, MF59@MDP exhibited a stronger capacity than the individual components to stimulate DC maturation in lymph nodes (Fig. 4H) and DC expression of the antigen-presenting molecules MHC-I (Fig. 4I) and MHC-II (Fig. 4J). This suggests that MF59@MDP has a greater ability to stimulate innate immune cell maturation and antigen presentation. As innate immune cells were enhanced, B cell responses showed proliferation of germinal center B cells in the lymph nodes (Fig. 4K). In the spleen, T cells exhibited induced proliferation of CD4 central memory T cells (CD4 T_{cm}) and effector T cells (CD4 T_{em}) (Fig. 4L&M). Among CD8 T cells, no significant difference was observed in CD8 central memory T cells (T_{cm}) (Fig. 4N), whereas CD8 effector T cells (CD8 T_{em}) proliferated markedly (Fig. 4O). Both *in vivo* and *in vitro*, DCs and lymph node cells exhibited trained immunity effects, with significant enhancement of DC number and phenotype in the lymph nodes. The enhanced phenotype of innate immune cells also exerted enhancing effects on adaptive cells such as B cells and memory T cells. In addition, the safety evaluation of the serum taken from the sacrificed mice was carried out. The liver function markers ALP and AST, the body's inflammatory marker CRP, and the renal function marker UREA were detected (Supplement Fig. 3A-D). The results showed that although these adjuvants cause limited immunogenic death in tissues, they will not cause damage to the body due to the high concentration of restriction and limited response.

2.5 MF59@MDP enhances T cell proliferation, IFN- γ cytokine secretion, DC maturation and antigen presentation by training immunity.

To determine whether innate immune cells in lymph nodes, following induction of trained immunity, can enhance adaptive immune cells and even ameliorate the immune bias of MF59, we administered PBS, MF59, or MF59@MDP as a trained immunity inducer in the first immunization. After MF59@MDP, which possesses training potential, had undergone in vivo training in mice and following a 28-day resting period after in vivo training, all mice were injected with MF59@MDP + CpG + DsCav1 as a second heterologous stimulus to examine innate immune cell phenotypes and adaptive immune cell proliferation. The results showed that in the mouse spleen, CD4 T cells and CD8 T cells proliferated markedly in the MF59@MDP group in which trained immunity had been induced (Fig. 5B&C). We then investigated whether antigen-specific T lymphocytes exhibited altered cytokine secretion. In IFN- γ ELISpot assays, MF59, as a Th2-biased adjuvant, reduced secretion of the Th1-type cytokine IFN- γ compared with the PBS group, whereas MF59@MDP with trained immunity enhanced IFN- γ production (Fig. 5D). No significant difference was observed between the two groups in the secretion of the Th2-type cytokine IL-4 (Fig. 5E). The overall IFN- γ /IL-4 ratio exhibited a gradually increasing trend (Fig. 5F). At the innate immune cell level, mice primed with MF59@MDP exhibited significantly upregulated DC maturation phenotypes (CD80, CD86) (Fig. 5G&H), and DC antigen-presenting capacity was also significantly enhanced (Fig. 5I).

2.6 CpG amplifies the training immune effect established by MF59@MDP on adaptive T cells.

Subsequently, to further amplify the trained immunity-mediated improvement in immune bias and the capacity to establish T-cell immune memory, we investigated the effects of different nanoemulsion adjuvants combined with CpG on immune bias in mice. After two immunizations, MF59@MDP + CpG enhanced Th1-type cellular immunity more effectively than MF59 + CpG (Fig. 6B), whereas Th2-type responses were not significantly increased (Fig. 6C), ultimately resulting in polarization of the cellular immune response in the mouse spleen toward a Th1 phenotype (Fig. 6D). In systemic cellular immune organs, the generation of memory T cells plays an important role in establishing durable immune protection. Notably, the generation of CD4 central memory T (T_{cm}) cells was significantly increased (Fig. 6E), and the generation of CD8 effector memory T (T_{em}) and central memory T (T_{cm}) cells was also markedly increased (Fig. 6F&G). These results indicate that, with the assistance of the CpG adjuvant, the ability of MDP to improve immune bias and establish central immunological memory was amplified.

2.7 MF59 @ MDP combined with CpG established effective virus protection in a mouse model.

Respiratory syncytial virus (RSV) is a virus that primarily infects elderly individuals with weakened immunity. Therefore, achieving virus-specific T-cell responses and establishing long-term protection through vaccination is of considerable importance for immunocompromised elderly populations. In our previous results, we demonstrated that, compared with MF59 + CpG + DS-Cav1, the MF59@MDP + CpG+DS-Cav1 vaccine enabled the establishment of superior CD4 Tcm and CD8 Tcm responses, providing an important prerequisite for the rapid development of effective antibody and cellular immunity upon re-exposure to the virus. We established a two-dose immunization regimen at a 28-day interval for the five vaccine groups, designated G1–G5. Subsequently, mice were rested for 63 days after the second immunization to ensure that both cellular and humoral immunity had declined to a resting state. Then, on day 91, each mouse was challenged with 5×10^5 PFU of the A2 virus strain in a challenge-protection experiment, and body weight and rectal temperature were measured daily (Fig. 7A). We found that mice in the PBS group exhibited a significant decrease in body weight (Fig. 8B) and a significant decrease in body temperature (Fig. 8C), and subsequently reached the ethical endpoint after body weight loss reached 20%. In the mouse spleen, we found that MF59@MDP + CpG+DS-Cav1 significantly increased the proliferation of antigen-specific cytotoxic T lymphocytes (Fig. 7D). HE-stained lung sections showed that the vaccine group reduced pulmonary inflammatory infiltration and effectively prevented virus-induced damage.

Discussion

Adjuvants are a critical component of successful vaccine development. Effective adjuvants can even determine the market availability and clinical application of a series of vaccines, such as the Flud influenza vaccine and the herpes zoster vaccine. MF59 is a Th2-biased immune adjuvant developed by Novartis in the 20th century that effectively enhances antibody-mediated immunity. Owing to its favorable biocompatibility and safety profile, MF59 has become an important adjuvant component in many vaccines. However, owing to its intrinsic mechanism of action, MF59 has several limitations, including a Th2 immune bias and insufficient induction of cellular immunity.

MDP is an agonist of the intracellular receptor NOD2. Upon binding to NOD2, MDP triggers a metabolic reprogramming signature characterized by upregulated glycolysis. Subsequently, oxidative phosphorylation (OXPHOS) and cholesterol synthesis can influence the transcriptional initiation of cytokine-encoding genes and alterations in histone acetylation and methylation, thereby modulating enhanced anti-infection immunity and immune damage.

Using engineered preparation technologies and referring to international formulations, we prepared MF59 with good particle size dispersion and an average particle size of approximately 170 nm. Comparison with domestic and international reference products confirmed that our preparation procedure was robust and that the adjuvant effect was comparable to that of the references.

Using this MF59 preparation platform, we rationally combined MDP with MF59. After injection, high local concentrations of Span85 and Tween80 induced immunogenic cell death at the injection site, releasing

large amounts of the “find-me” signal ATP and the immunogenic substance dsDNA. These signaling molecules acted on tissue-resident monocytes, inducing the production of chemokines such as CCL2, CCL3, and CCL4, which in turn promoted antigen-presenting cells to phagocytose nanoparticles and migrate to draining lymph nodes. After MF59@MDP was taken up by DCs, monocytes, macrophages, and other cells, MDP was released and bound to intracellular NOD2 receptors, thereby stimulating DC maturation and metabolic reprogramming. Activated DCs further promoted T cell activation and proliferation. In both in vivo and in vitro trained immunity experiments, we demonstrated that MF59@MDP enhanced the secretion of the inflammatory cytokines IL-6 and TNF- α . In combined antigen immunization, MF59@MDP exhibited clear cellular immune advantages in DC activation, antigen-presenting molecule expression, and T cell activation and proliferation.

To amplify this trained immunity effect, we introduced the CpG adjuvant and demonstrated the advantages of the combined adjuvant in both immune bias and memory T cell proliferation.

Finally, in BALB/c mice challenged with respiratory syncytial virus, the vaccine groups showed no significant decreases in body weight or body temperature, indicating that the high levels of binding antibodies induced by the adjuvants could neutralize viral challenge. Notably, CTLs were significantly upregulated in the MDP-containing adjuvant group, consistent with the function of MDP in stimulating T cell immunity. Mice in the MF59@MDP + CpG+DS-Cav1 group, which exhibited high humoral and cellular immunity, showed the least pronounced lung injury, indicating that dual activation of humoral and cellular immunity is more likely to protect the host against viral infection. However, this study still lacks an analysis of epigenetic changes in DCs in draining lymph nodes after trained immunity induction, which relies on more refined omics analyses. Although we evaluated the impact of trained immunity in non-central lymphoid organs on antiviral effects by challenging mice after a prolonged 64-day resting period, this assessment remains insufficiently detailed. Future studies are needed to determine the translational value and advantages of inducing trained immunity in peripheral lymphoid organs.

Material and methods

3.1 Experimental animals and models

The animals used in this study were SPF female BALB/c mice (6–8 weeks old). They were purchased from Sberford (Beijing) Biotechnology Co., Ltd., and the mice were raised in the Experimental Animal Center of the Institute of Medical Biology, Chinese Academy of Medical Sciences. All animal experiments were in accordance with the ethical and moral standards of experimental animals. The experiment was approved by the Animal Use and Management Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences (ethical number : DWSP202110005).

3.2 Preparation of MF59 and MF59@MDP nanoemulsion adjuvant

Referring to the formula of MF59 nanoemulsion adjuvant reported by Novartis Pharmaceutical Co., Ltd^[51], we prepared a nanoemulsion adjuvant formula with a mass volume ratio (w/v) of 4.3% squalene, 0.5% Span85, 0.5% Tween80 and dissolved in citric acid buffer (pH6.5 ~ 7.0). MF59 is composed of 4.3% (w/v) squalene and 0.5% (w/v) Span85 in a beaker, and the mixed oil phase is uniformly mixed at 1000 rpm. MM59@MDP was composed of 4.3% (w/v) squalene, 0.5% (w/v) Span85 and 3 mg MDP dissolved in 1mL DMSO in a beaker. The mixed oil phase was uniformly mixed at 1000 rpm. 0.5% (w/v) Tween80 and citric acid buffer (pH 6.5 ~ 7.0) were mixed in a conical flask, and the mixed aqueous phase was uniformly mixed at 1000 rpm. The mixed solution was uniformly mixed at 1000 rpm for 30 min to prepare the colostrum. The nanoemulsion adjuvant was prepared at 700 bar at high pressure homogenizer. After the preparation was completed, the 0.22 µm filter membrane was filtered to obtain sterile MF59 nanoemulsion adjuvant under different conditions.

3.3 The MF59 nanoemulsion adjuvant prepared under different conditions was detected by Malvern particle size analyzer.

After 10 µL of MF59 nanoemulsion particles prepared by different engineering methods were diluted to 1 mL with PBS, the cuvette was placed in a Malvern particle size analyzer for dynamic light scattering (DLS) measurement to determine the particle size distribution (size) and polydispersity index (PDI) of different nanopreparations.

3.4 Transmission electron microscopy (TEM) was used to detect the adjuvant prepared under different conditions.

10 µL of nanoemulsion particles prepared under different conditions were added to the carbon-coated copper mesh, and then stood at room temperature for 5min. The excess liquid on the copper mesh was removed by absorbent paper, and the 2% phosphotungstic acid solution (pH = 6.5) was prepared. The staining solution was dried for 30 min, and the prepared electron microscope samples were placed in a transmission electron microscope (model HITACHI H-7650) for observation at a voltage of 80 kV.

3.5 Nanoemulsion adjuvant physically stimulates cell death to release immunogenic substances

293T cells were seeded proportionally in 6-well plates. When confluence reached 80%, a total volume of 100 µL was prepared, to which different volumes of MF59@MDP were added, with the remaining volume made up using complete medium. After 24 h of incubation, the culture supernatants were collected for immunogenicity detection. ATP, LDH, and dsDNA were measured using (Beyotime cat: S0027), (Beyotime cat: C0019S), and (Yeasen cat: 12640ES60), respectively.

3.6 Isolation and preparation of mouse BMDCs

The femur and tibia of mice were taken : C57BL/6J mice aged 6–8 weeks were anesthetized and sacrificed, and the mice were immersed in 75% alcohol for disinfection. An incision of about 1cm in

diameter was cut in the abdomen of the mice with scissors. The femur and tibia of the mice were taken out, and the muscles around the bone were shaved as much as possible and immersed in 75% alcohol for 5min. The isolation of bone marrow cells : The isolated femur and tibia of mice were transferred to a sterile PBS. After washing 2–3 times, the ends of the femur and tibia were cut with scissors. The bone marrow cells in the femur and tibia were repeatedly washed with 1mL sterile RPMI-1640 medium, and the collected cell suspension was placed in a 15mL centrifuge tube using a 70mm cell network filter. The collected cell suspension was centrifuged at 500 xg for 5min, 5mL of red blood cell lysate was added to lyse red blood cells, and then 5mL of sterile PBS was added to terminate, 800 xg was centrifuged for 5min, and the cells were collected. Induced differentiation of BMDCs: The cells were resuspended in RPMI-1640 complete medium containing 10% fetal bovine serum and 1% double antibody, and then added to the culture dish, 10mL/dish. Recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF) was added to the culture medium to a final concentration of 10 ng/mL. The culture dish was placed in a 37°C, 5% CO₂ incubator for static culture. Half of the medium was changed 3 days after culture. 5mL medium was slowly sucked out, and RPMI-1640 complete medium containing 10ng/mL GM-CSF was added to continue culture (the medium was changed every two days). BMDCs were collected after 8days of culture for subsequent experiments.

3.7 Western Blot Analysis

BMDCs were isolated as reported previously. A total of 1×10^6 BMDCs were transfected with 400μL MF59@12μg of MDP@400μL MF59@MDP. To analyze trained immunity pathway activation, cells were lysed at 2 hours post-incubation using RIPA buffer containing PMSF.

Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore), and probed with the following primary antibodies: anti-AKT (CST, 4691T, 1:1000), anti-Phospho-AKT (Ser473) (CST, 4060T, 1:1000), anti-mTOR (CST, 2983T, 1:1000), anti-Phospho- mTOR (Ser2448) (CST, 5536T, 1:1000), anti-HIF-1α (CST, 14179T, 1:1000), and anti-β-Actin (Proteintech, 66009-1-Ig, 1:20000). HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Proteintech, 1:5000) were used, and signals were detected using an Omni-ECL™ ultrasensitive chemiluminescence kit (Epizyme Biotech).

3.8 Supervise the stimulating effect of immunogenic substances on DC cells.

The collected cell death supernatant was mixed with the complete medium at a ratio of 1:4, and the stimulated differentiated BMDC was laid into a 96 U-shaped hole. The mixed medium was incubated with BMDC overnight. After 24 hours of stimulation, the medium was removed for flow cytometry staining. The phenotypic and functional changes of DC cells after stimulation were identified by flow cytometry.

3.9 immune animal

The purified pre-F protein was diluted to 200μg/mL or 400μg/mL, and 25μL diluted protein solution was mixed with 25μL nanoemulsion adjuvant to prepare pre-F protein subunit vaccine. The vaccine was

intramuscularly injected into BALB/c mice according to the immune flow chart, and blood was collected after immunization. The antibody level was detected by ELISA.

3.10 The titer of IgG binding antibody against per-F protein was analyzed by ELISA.

The 50ng pre-F subunit protein was diluted with ELISA coating solution and placed in an ELISA plate, 50 μ L per well, coated overnight at 4°C, and washed three times with PBST buffer. The prepared 2% BSA was added, 200 μ L per well, incubated at 37°C for 1–2 h, and washed with PBST buffer for 3 times. Diluted serum was added, 50 μ L per well, incubated at 37°C for 1h, and washed with PBST buffer for 3 times. The prepared HRP-Anti Mouse IgG secondary antibody was added, 50 μ L per well, incubated at 37°C for 1h, and washed 5 times with PBST buffer. Add the chromogenic solution TMB, and stop reading the OD value at an appropriate time. When the OD value is greater than the OD value of the blank group*2.1, it is considered to be a positive hole.

3.11 Tissue Section Immunofluorescence

Mice were intramuscularly injected with the following Adjuvant: PBS, MF59(25 μ L), MDP(750ng), MF59@MDP(25 μ L), Muscle tissue was collected 72 hours after injection. Tissue sections were incubated overnight at 4°C with primary antibodies: anti-CD11c (Servicebio, GB11059, 1:2000). Sections were then incubated with Alexa Fluor 488 conjugated secondary antibodies for 1 hour at room temperature, counterstained with DAPI (Servicebio, G1012), and imaged using a Nikon Eclipse C1 fluorescence microscope.

3.12 Isolation of mouse spleen lymphocytes in vitro

After the mice were sacrificed, the spleen was quickly removed from the body and collected into a centrifuge tube. The spleen was ground in a 70 μ m filter in a biological safety cabinet, and the lymphocyte separation solution was added several times to grind until the final volume was 5 mL. Then, 500 μ L RPMI-1640 medium was slowly added along the tube wall to ensure the separation of the two interfaces. After centrifugation at 800 xg for 30min, the white middle layer, namely the lymphocyte layer, was slowly taken out and sucked out into another centrifuge tube. If the middle layer was red, it indicated the presence of red blood cells. The red blood cell lysate was used to lyse the red blood cells, and then centrifuged at 500 xg for 5min to collect the precipitated lymphocytes. After the supernatant was discarded, an appropriate amount of 1640 complete medium was used to re-suspend the cells, and then the cells were counted.

3.13 Isolation of mouse lymph node lymphocytes in vitro

After sacrifice, the lymph nodes were removed and placed in a 1.5mL centrifuge tube on ice. In the centrifuge tube, the lymph nodes were chopped with scissors, and 500 μ L of collagenase A solution (1mg/mL) was added in RPMI-1640 medium. The centrifuge tube was placed on a shaker at 37°C, 120rpm, and the lymph node tissue was digested for 1h ; the subsequent operation was basically consistent with the operation of isolating mouse spleen lymphocytes in vitro.

3.14 Detection of cellular immune level by flow cytometry

Surface staining: 96-well U-shaped plates were centrifuged at 500 xg at room temperature for 5 min, and cells were washed twice with cell staining buffer, 2min each time. Cells were resuspended with 100µL cell staining buffer, and the corresponding surface antibody mixture was added to each well and mixed. After incubation for 30min at room temperature, 200µL cell staining buffer was added to each well and washed once. If intracellular staining was not required, 200µL cell staining buffer was used for resuspension and detection. Cells were plated and stained : 1×10^7 lymphocytes were plated on 96-well U-shaped plates. Stimulation (such as cytotoxic T lymphocytes): pre-F antigen protein was added to the plate for stimulation (final concentration of 50µg/mL). After 12h of stimulation in the incubator, 1µL of 100 x Brefeldin A was added to each well, and BFA was terminated, and then cultured for 2h. Cell fixation : 200µL of 1x Fixation buffer was added to each well to re-suspend the cells, and incubated at room temperature in dark for 20min. Subsequently, the fixative was removed by centrifugation at 500 xg for 5min, and 200µL cell staining buffer was washed once, and the supernatant was discarded. Cell membrane rupture : Dilute 10x Permeabilization Wash Buffer with high-pressure distilled water, add 200µL 1x Permeabilization Wash Buffer to re-suspend fixed cells, 500 xg, centrifuge for 5min, discard the supernatant. The cells were resuspended with 200µL cell Staining buffer for detection.

3.15 The level of secreted cytokines was detected by enzyme-linked immunospot assay (ELISPOT).

Pretreatment : Mouse IFN-γ / IL-4 pre-coated capture antibody plates were washed four times with 200µL/well PBS, followed by incubation with 200µL/well 1640 complete medium at room temperature for 30min. Cell inoculation: After the mouse lymphocytes were isolated and counted, the 96-well cell plate was activated with RPMI-1640 medium, and then the cells were inoculated. Each well was inoculated with 100 µL cell suspension (3×10^5 cells) ; stimulator stimulation: The concentration of pre-F protein was added to the experimental well at a final concentration of (10µg/mL). Incubation at 37°C, 5% CO2 incubator for 24h ; subsequently, the cells were washed with 200µL/well PBS for 5 times. Incubation detection antibody: FBS was diluted with PBS to 0.5%, and 0.5% FBS-PBS diluent was prepared. The diluent was diluted to a concentration of 1µg/mL, 100µL/well, and incubated at room temperature for 2h. Then washed with 200µL/well PBS for 5 times. Incubation of ALP: The antibody was diluted with 0.5% FBS-PBS to 1µg/mL, 100µL/well, and incubated at room temperature for 1h. Then washed with 200µL/well PBS for 5times. Color development and termination: The developer was filtered with a 0.45µm filter, and 100µL of color development was added to each hole for 20 ~ 45 min. The base could be removed when spots appeared in the positive hole, and the color development was terminated by washing with tap water. Place the board in a cool place at room temperature and let it dry naturally. ELISPOT reader system was used to count spots, and various parameters of spots were recorded for statistical analysis.

3.16 Mice challenge protection experiment

The mice were intranasally inoculated with 5×10^5 PFU A2 strain virus at 56 days after two doses of immunization. The body weight and body temperature of the mice were monitored daily after challenge, and the mice were sacrificed at 4 days after challenge to evaluate the pathological changes of the lungs.

3.17 Bronchoalveolar lavage fluid and left lung of mice were taken.

The indwelling needle containing 1 mL PBS solution was used for lung lavage, and the alveolar lavage fluid was obtained after repeated blowing and sucking for three times, and the volume of the lavage fluid was recorded. The supernatant and cells containing the lavage fluid were obtained by centrifugation at 500 xg for 5 min. The cells were blown with the same volume of PBS. One part was counted using a red blood cell counting plate, and the other part was taken 10 μ L for flicking and Giemsa staining. After the left lung was separated, 4% paraformaldehyde was placed for fixation and HE sections were performed.

3.18 Hematoxylin and eosin staining of mouse lung tissue

Paraffin sections were dewaxed to water: The sections were placed in an environmentally friendly dewaxing solution for 20 min—environmentally friendly dewaxing solution for 20 min—anhydrous ethanol for 5 min—anhydrous ethanol for 5 min—75% alcohol for 5 min, and washed with tap water. Rewarming fixation of frozen sections: The frozen sections were taken out from the refrigerator at -20°C and restored to room temperature, fixed with tissue fixative for 15 min, and rinsed with running water. Pretreatment: Slices were treated with high-definition constant staining pretreatment solution for 1 min. Hematoxylin staining: Slices were stained with hematoxylin staining solution for 3–5 min, washed with tap water, differentiated with differentiation solution, washed with tap water, returned blue solution returned blue, and washed with running water. Eosin staining: The sections were dehydrated in 95% alcohol for 1 min and stained in eosin staining solution for 15 s. Dehydration sealing: slices were placed in anhydrous ethanol 2 min—anhydrous ethanol 2 min—anhydrous ethanol 2 min—n-butanol 2 min—n-butanol 2 min—xylene 2 min—xylene 2 min transparent, neutral gum sealing. Microscopic examination, image acquisition and analysis.

3.19 statistical analysis

Statistical analysis was performed using prism 9.0 (GraphPad software). The values were shown as mean $\pm$ SD. One-way or T-test analysis was used for comparison between two or more groups. $P < 0.05$ was considered to be significantly different (* $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), and 'ns' indicated no significant difference.

Declarations

Competing interests:

The authors declare no competing interests.

Data and materials availability

Requests for resources and reagents should be directed to BSR. Source data are provided with this paper. Supporting Information is available from the Wiley Online Library or from the author.

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Author Contribution

BSR, MZZ and JRH. initiated and coordinated the project. Animal experiments, analyzed the data, and drafted the manuscript: BSR, MZZ. Animal experiments and flow cytometry analysis: JRH, QL, HMB, YY, YTD, XNL, WJS, PZ, TY, XY, HL. Technical support: JRH. Designed the research, provided guidance and supervision for this study, and reviewed the manuscript: YBM

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Figures

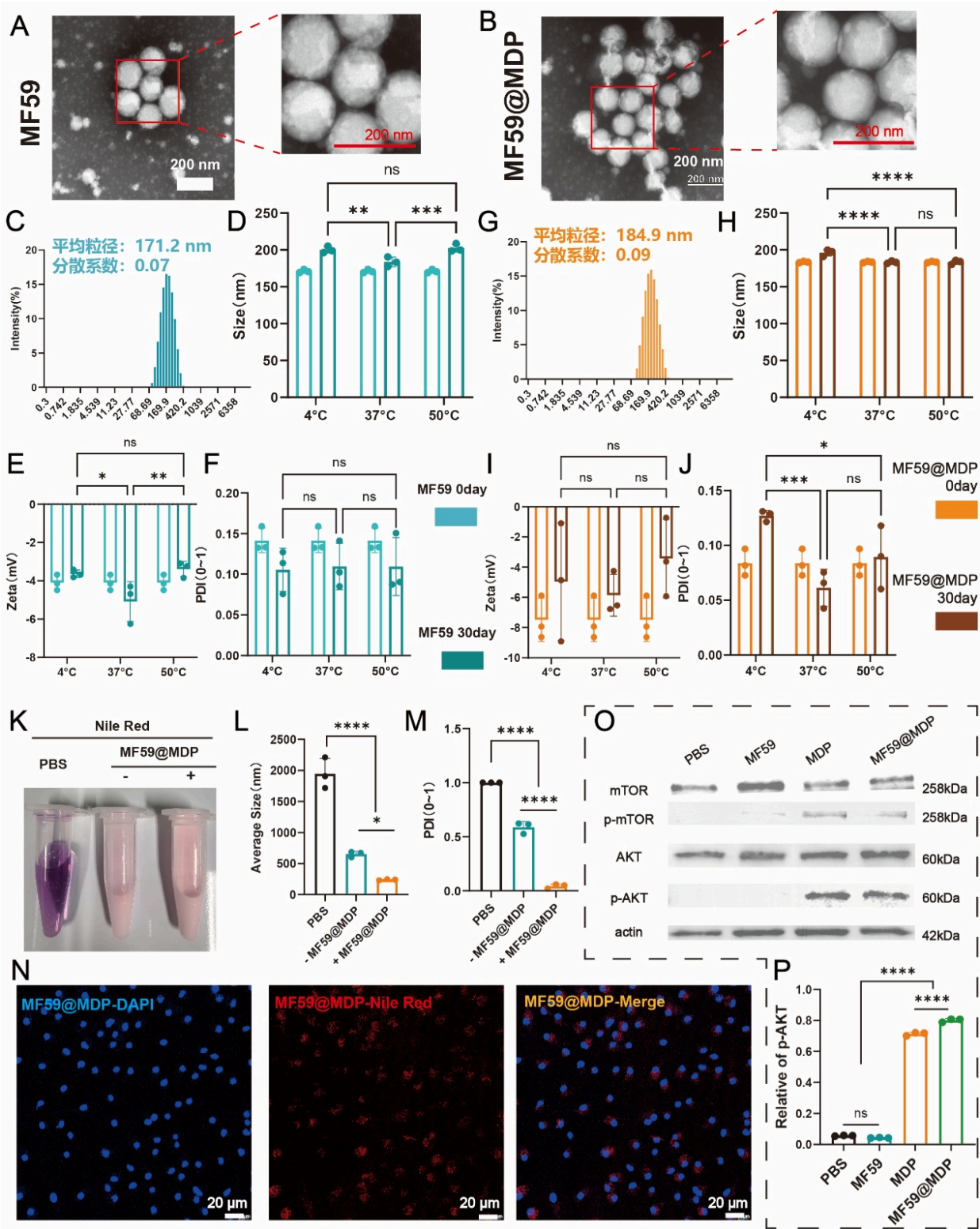


Figure 1

The morphology, physical and chemical properties of MF59@MDP were characterized.

(A) TEM images and enlarge figure of MF59 (scale bar: 50 μ m).

- (B) TEM images and enlarge figure of MF59@MDP (scale bar: 50 μm).
- (C) Particle size and dispersion coefficient of MF59
- (D) The particle size changes of MF59 after 30 days of storage at different temperatures.
- (E) Zeta change.
- (F) PDI change.
- (G) Particle size and dispersion coefficient of MF59@MDP
- (H) The particle size changes of MF59@MDP after 60 days of storage at different temperatures.
- (I) Zeta change.
- (J) PDI change.
- (K) The apparent changes of MF59@MDP with Nile Red dye before and after high pressure homogenization (PBS: 1 mL PBS+1 μmol Nile Red, -MF59@MDP: 1 mL MF59@MDP+1 μmol Nile Red non-nanometerization, +MF59@MDP: 1 mL MF59@MDP+1 μmol Nile Red nanometerization).
- (L) Particle size comparison
- (M) PDI comparison
- (N) The uptake of Nile Red-labeled MF59@MDP by BMDC cells was detected by confocal fluorescence microscopy. After incubation for 24 h, the cells were washed repeatedly to remove the unengulfed fluorescently labeled particles and then imaged (scale bar: 20 μm).
- (O) Activation of signaling pathways after stimulation with different adjuvants and BMDC.
- (P) Upregulation of p-AKT.

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (D-F, H-J, L&M, P). Significance is denoted as follows: ns, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

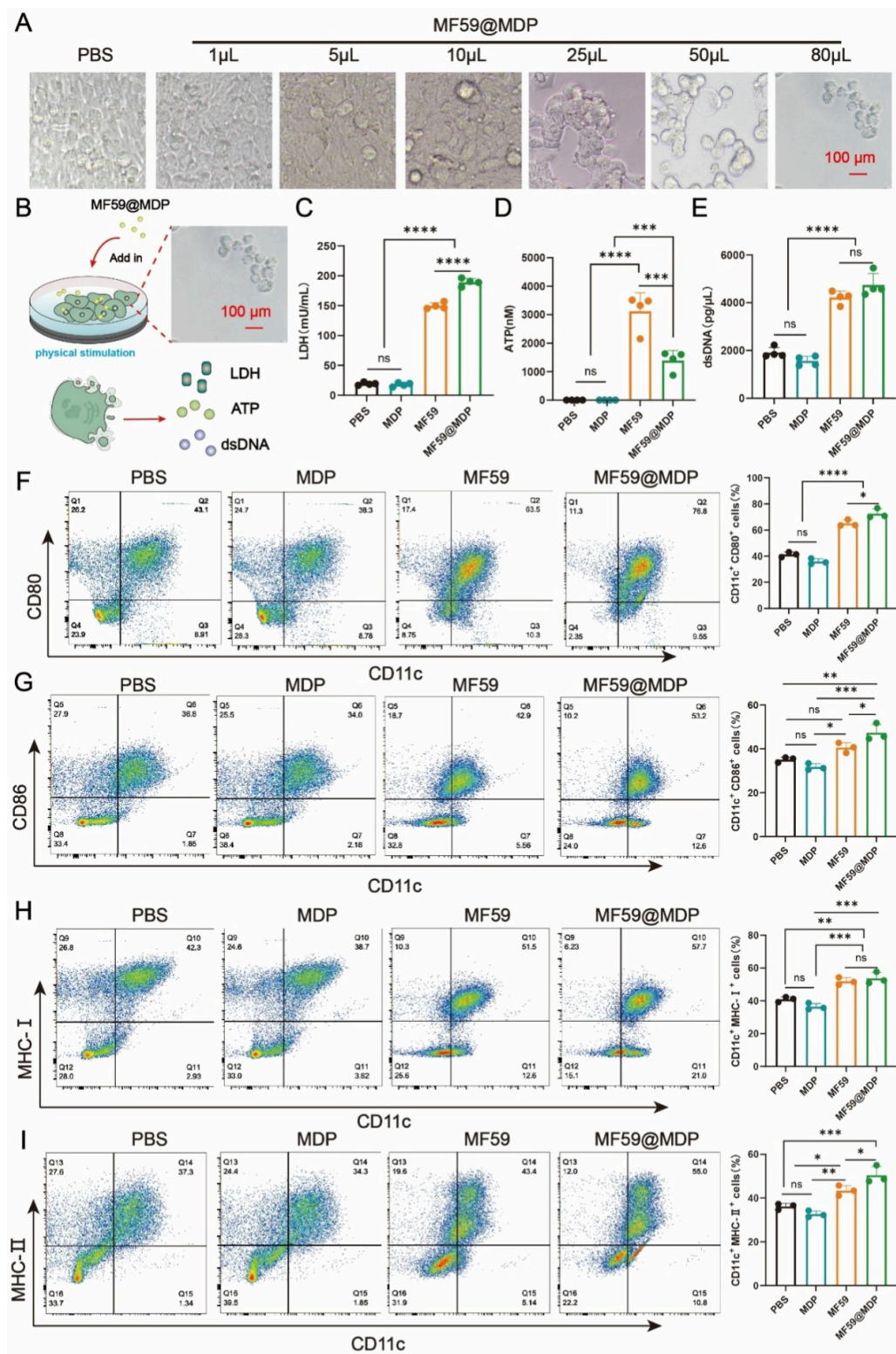


Figure 2

Immunogenic death and adjuvant effect evaluation of MF59@MDP adjuvant

(A) MF59@MDP dose-dependently physically stimulated cell death.

(B) A schematic diagram of MF59@MDP stimulation causing cell death to release immunogenic substances.

(C) LDH level released by 293T after incubation with adjuvant for 24h.

(D) ATP content.

(E) dsDNA content.

(F) The expression level of CD80 mature marker was stimulated after the cell culture supernatant was incubated with BMDC.

(G) The expression level of CD86.

(H) The expression level of MHC-I.

(I) The expression level of MHC-II.

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (C-I). Significance is denoted as follows: ns, not significant ($P > 0.05$); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

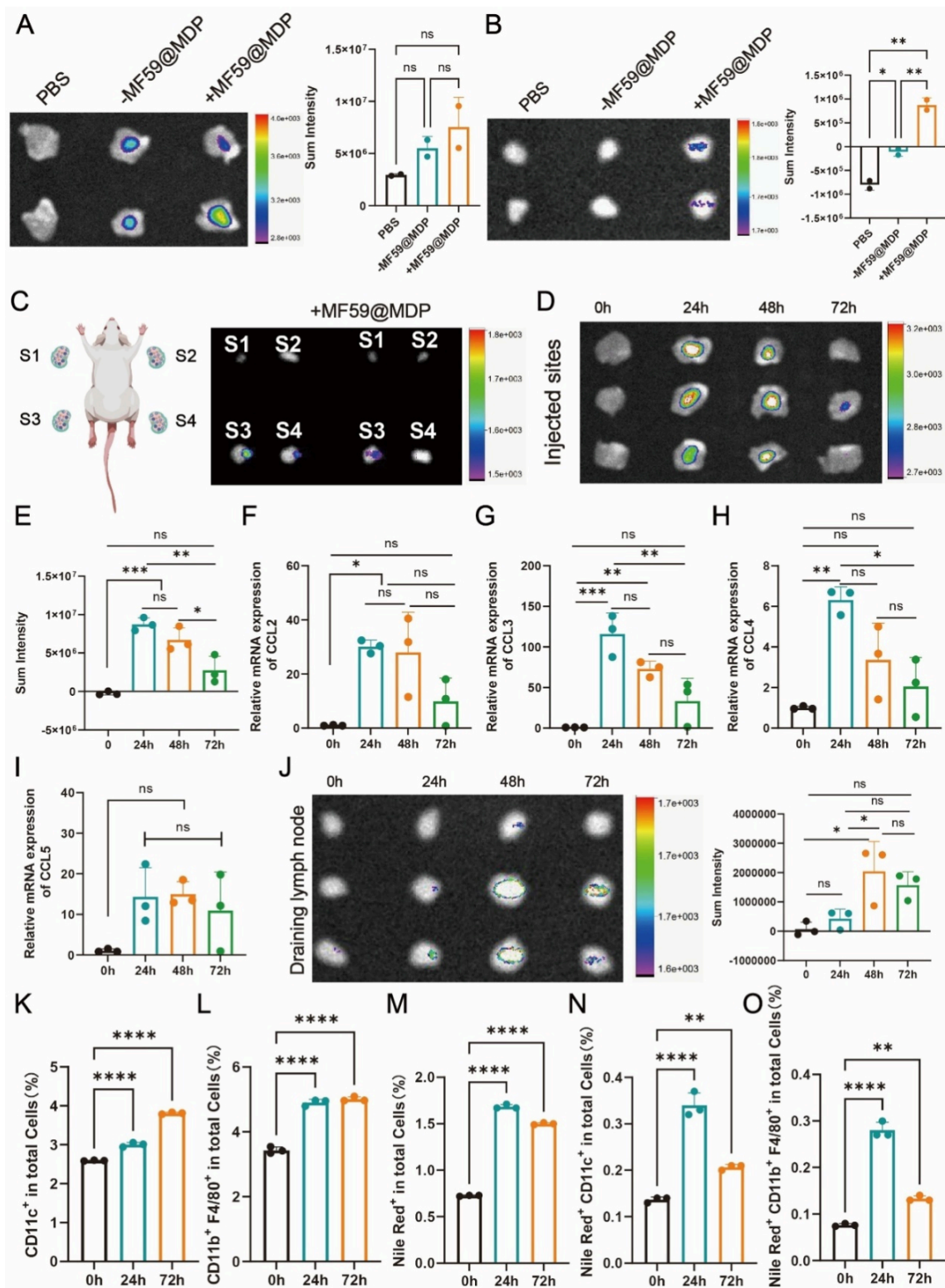


Figure 3

The kinetic changes of MF59@MDP in the injection site and draining lymph nodes.

(A) The enrichment of MF59@MDP in the injection part before and after nanocrystallization (PBS: 1 mL PBS+1 μ mol Nile Red, -MF59@MDP: 1 mL MF59@MDP+1 μ mol Nile Red non-nanometerization, +MF59@MDP: 1 mL MF59@MDP+1 μ mol Nile Red nanometerization).

- (B) Distribution of draining lymph nodes.
- (C) Comparison of enrichment of MF59@MDP in various lymph nodes of the whole body.
- (D) The kinetic changes of MF59@MDP at the injection site.
- (E) The statistical values of MF59@MDP at the injection site.
- (F-I) The up-regulation of CCL2 gene in the injection site of MF59@MDP(F). CCL3(G). CCL4(H). CCL5(I).
- (J) The dynamic changes and statistical values of MF59@MDP in the drainage lymph nodes.
- (K-O) The proportion of CD11b⁺ cells in draining lymph nodes(K). CD11b⁺ F4/80⁺ cells(L). Nile Red⁺ cells(M). Nile Red⁺ CD11b⁺ cells(N). Nile Red⁺ CD11b⁺ F4/80⁺ cells(O).

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (A&B, E-O). Significance is denoted as follows: ns, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

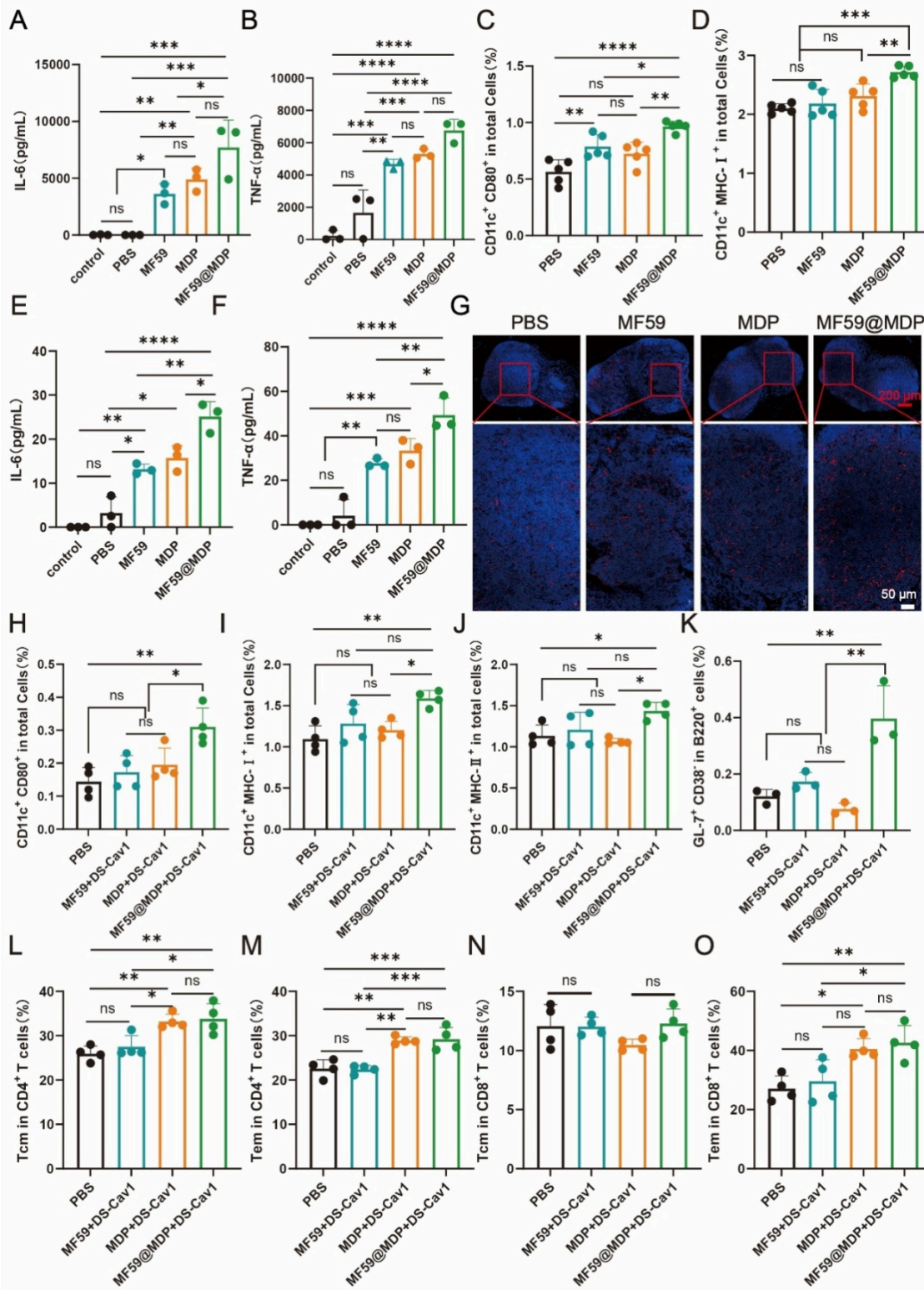


Figure 4

MF59@MDP induced the training immunity of DC in draining lymph nodes and enhanced the proliferation of B cells and T cells.

(A) Heterologous stimulation of IL-6 secretion with LPS after induction of different nanoparticles.

- (B) TNF secretion in the supernatant.
- (C) CD80 expression in draining lymph nodes of mice.
- (D) The expression of MHC-I.
- (E) Lymphocytes in draining lymph nodes were stimulated with LPS to secrete IL-6.
- (F) TNF- α secretion.
- (G) The enrichment of DC in draining lymph nodes.
- (H) The expression of DC maturation marker CD80 in lymph nodes.
- (I) The expression level of MHC-I.
- (J) The expression level of MHC-II.
- (K) The proportion of GC B cells in lymph nodes.
- (L) Proportion of CD4 Tcm positive cells in spleen.
- (M) CD4 Tem.
- (N) CD8 Tcm.
- (O) CD8 Tem.

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (A-F, H-O). Significance is denoted as follows: ns, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

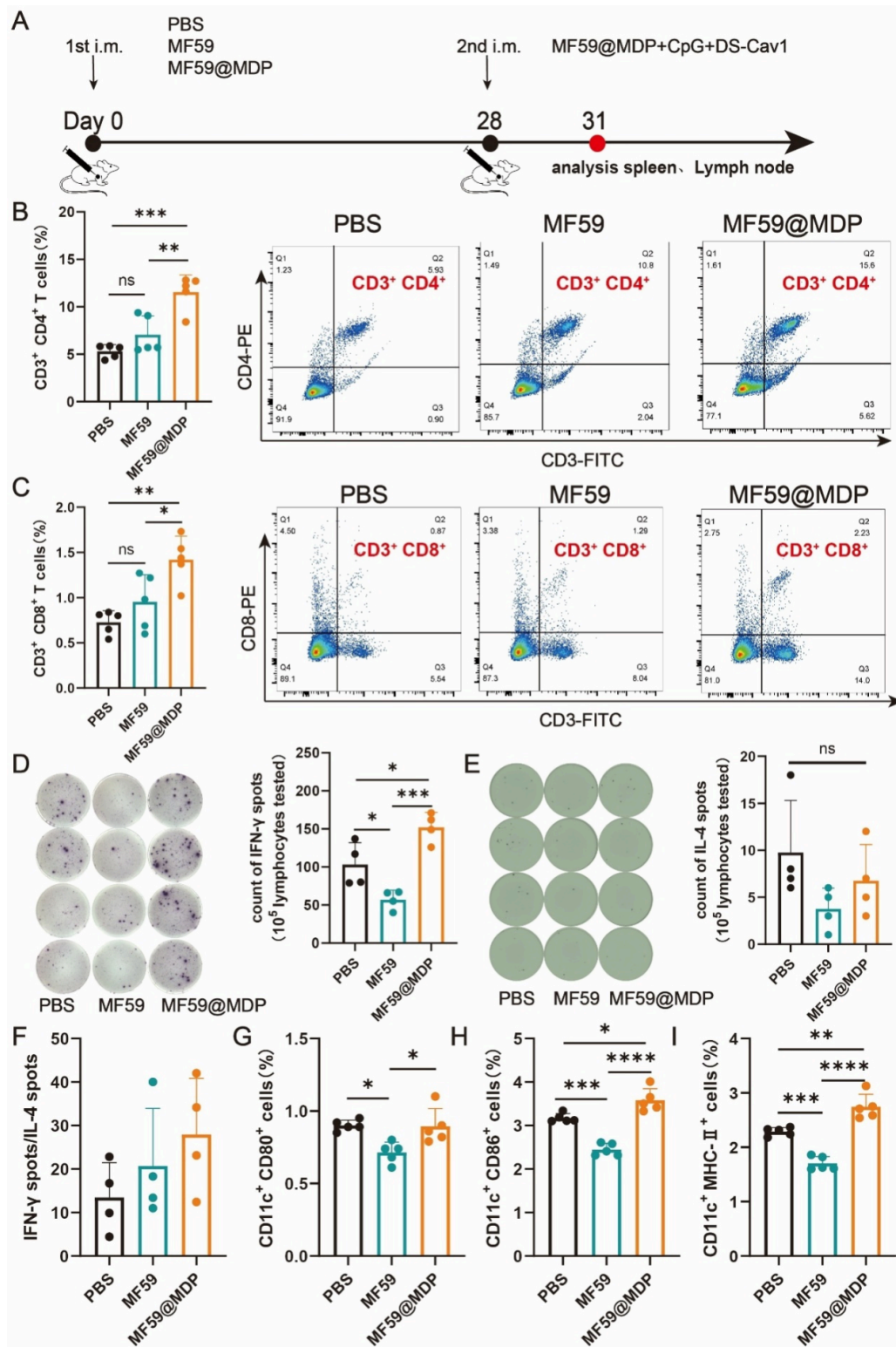


Figure 5

The effect of MF59@MDP on innate immune cells and adaptive immune cells by training immunity.

(A) Vaccine immunization program.

(B) The gate strategy and proliferation of CD3⁺ CD4⁺ in spleen.

(C) CD3⁺ CD8⁺ in spleen.

(D) RSV preF antigen-specific T cells secreted IFN- γ spot diagram and statistical value.

(E) IL-4 spot diagram and statistical value.

(F) IFN- γ spot/ IL-4 spot

(G) The expression of CD11c⁺ CD80⁺ in draining lymph nodes was up-regulated after two-needle immunization.

(H) CD11c⁺ CD86⁺.

(I) CD11c⁺ MHC-II⁺.

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (B-I). Significance is denoted as follows: ns, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

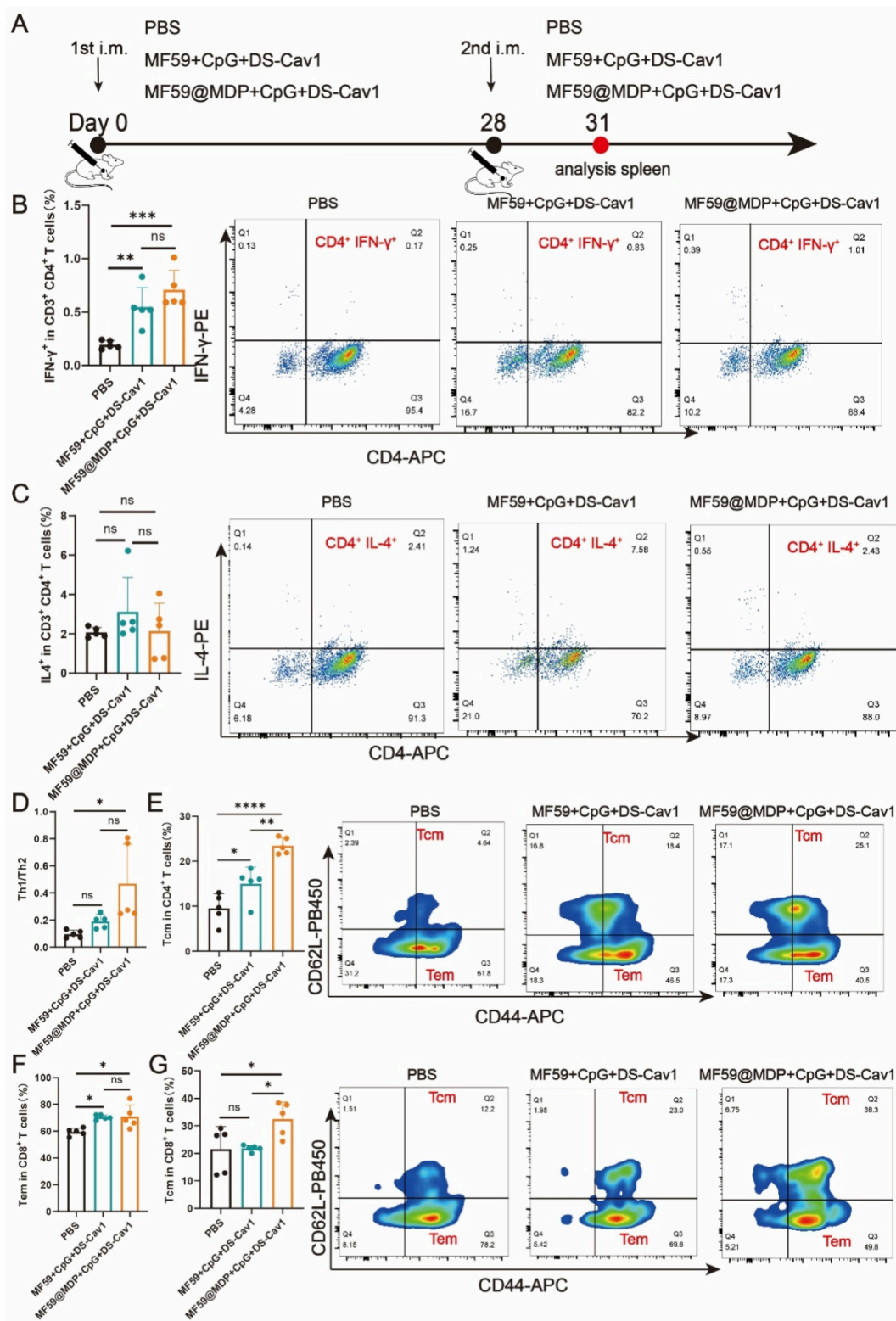


Figure 6

CpG enhances the effect of MF59@MDP-induced training immunity on adaptive T cells.

(A) Vaccine immunization program.

(B) RSV preF antigen-specific T cells secreted IFN- γ spot diagram and statistical value.

(C) IL-4 spot diagram and statistical value.

(D) The ratio of Th1 and Th2.

(E) CD4 Tcm (CD4⁺ CD44⁺ CD62L⁺) in spleen.

(F) CD8 Tem (CD8⁺ CD44⁺ CD62L⁻) in spleen.

(G) CD8 Tcm (CD8⁺ CD44⁺ CD62L⁺) in spleen

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (B-G). Significance is denoted as follows: ns, not significant ($P > 0.05$); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

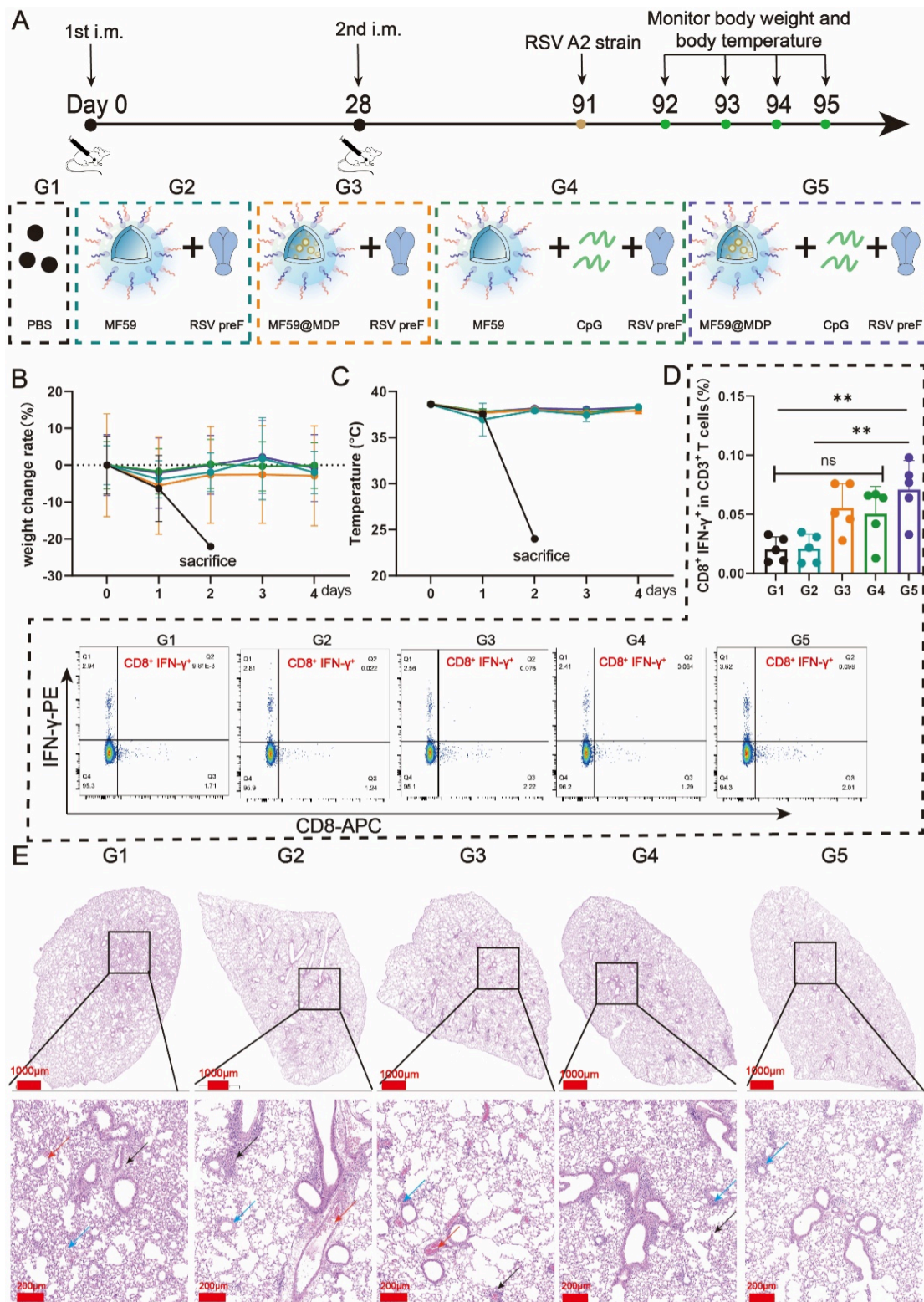


Figure 7

Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test (D). Significance is denoted as follows: ns, not significant ($P > 0.05$); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Physiological and pathological characteristics of BALB/c in challenge model.

- (A) Vaccine and challenge immunization program.
- (B) The relative change rate of body weight of BALB/c mice after challenge.
- (C) The body temperature change rate of BALB/c mice.
- (D) The proportion circle strategy of CTL ($CD3^+ CD8^+ IFN-\gamma^+$) cells in the spleen of mice.
- (E) HE pathological sections of mouse lungs.

Supplementary Files

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- [supplementinformation.docx](#)
- [floatimage1.jpeg](#)