

15 **Abstract:**

16 Aluminum adjuvants have been used for a century in various vaccines due to its ability to
17 potentiate humoral immunity and safety records since 1920s. Manganese is an essential
18 micronutrient required for diverse biological activities in cells. We previously found that Mn^{2+} is a
19 strong type I-interferon stimulator activating the cGAS-STING pathway. Herein we report that a
20 colloidal manganese salt (MnJ) is a potent adjuvant to induce both humoral and cellular immune
21 responses, particularly CTL activation. When administrated intranasally, MnJ was also a strong
22 mucosal adjuvant, inducing high levels of IgA antibodies. MnJ strongly promoted dendritic cell
23 maturation and antigen-specific T cell activation. Interestingly, IL-1/-18 induction and release by
24 Mn^{2+} -activated ASC-mediated inflammasomes were not observed. MnJ showed great adjuvant
25 effects to all tested antigens including inactivated viruses, recombinant proteins and peptides by
26 either intramuscular or intranasal immunization. These findings may have implications in
27 developing potent but safe Mn^{2+} -containing vaccines.

28 **Main Text:**

29 Vaccination is one of the most successful public health interventions. Adjuvants are widely used to
30 increase the immunogenicity of vaccines with various advantages: (1) reducing the amount of
31 antigens; (2) reducing the number of immunizations; (3) inducing a faster protection; (4)
32 improving the efficacy of vaccines in newborns, the elderly or immunocompromised populations
33 ^{1,2}. Over the past few decades, large amounts of adjuvants have been developed. According to their
34 different mechanisms of action, adjuvants are divided into two categories: immune potentiators and

35 delivery systems. Some immune potentiators, composed of pathogen-associated molecular patterns
36 (PAMPs) or synthesized activators of pattern-recognition receptors (PPRs), activate innate immune
37 responses to induce the subsequent production of cytokines and chemokines. Other potentiators,
38 composed of cells or cytokines like dendritic cells, IL-12 or GM-CSF, directly activate immunity.
39 Delivery systems including liposome, micelles, virosome, nanoparticles, microsphere, Oil/Water
40 emulsion, virus-like particles (VLP) and immune stimulating complexes (ISCOM), usually carry
41 antigen to target cells and assist antigen uptake by antigen presenting cells (APCs) ^{3,4}.

42 The immune enhancement effect of aluminum salts (Alum) was first reported by Glenny et al. in
43 the 1920's when they found that injection of guinea pigs with diphtheria toxoid precipitated with
44 potassium aluminum provided greater protection than toxoid alone ⁵. Since then, Alum-containing
45 adjuvants have been employed in billions of doses of vaccines and administered annually to
46 millions of people ⁶. In fact, Alum is the only human adjuvant widely used, partly due to its
47 minimal reactogenicity and inexpensiveness ⁷. However, Alum-adjuvanted vaccines need repeated
48 administrations which may cause or manifest its adverse effects including increased IgE production
49 and neurotoxicity ⁸. In addition, Alum mainly induces T helper 2 (TH2) cell response through
50 NLRP3 inflammasome activation ⁹⁻¹¹, but not TH1 or cytotoxic T-lymphocyte (CTL) response ^{1,12}.
51 Therefore, Alum is generally believed to be unable to elicit cellular immune responses that are
52 essential for virus or tumor vaccines.

53 In the past decades, however, the medical need for new adjuvants is increasing as (1) the
54 tremendously increased use of purified antigens like recombinant proteins with low

55 immunogenicity due to the absence of immunostimulatory components recognized by PRRs; (2)
56 adjuvants inducing cellular immune responses especially CTL are badly needed for virus and
57 cancer vaccines¹³⁻¹⁵. So far few adjuvants have been approved by the US Food and Drug
58 Administration for use in humans and several formulations are in clinical trials. The oil-in-water
59 MF59 in influenza vaccines for the elderly was approved in the 1990's, followed by AS03 in
60 vaccines against avian influenza virus, AS04 in hepatitis B virus (HBV) and human papillomavirus
61 (HPV) vaccines, and AS01 in herpes zoster virus vaccines¹⁶. Toll-like receptor (TLR) agonists,
62 like the CpG DNA and poly (I:C), have been studied in the past two decades as new adjuvant
63 candidates⁷. cGAS-STING¹⁷⁻²⁰ agonists like DMXAA²¹, c-di-GMP²², cGAMP²³ and Chitosan²⁴
64 also showed some adjuvant effects.

65 Manganese (Mn) is a nutritional inorganic trace element required for a variety of physiological
66 processes including development, reproduction, neuronal function and antioxidant defenses^{25,26}.
67 Mn (Mn²⁺ in general cases) is essential for some metalloenzymes such as Mn superoxide dismutase
68 (SOD2, Mn³⁺ or Mn²⁺ in this case), glutamine synthetase (GS), and arginase²⁷. However, its
69 function in regulating immunity is largely unknown. Previously we found that Mn²⁺ was required
70 for the host defense against DNA virus by increasing the sensitivity of the DNA sensor cGAS and
71 its downstream adaptor protein STING. Mn²⁺ was released from mitochondria and Golgi apparatus
72 upon virus infection and accumulated in the cytosol where it bound directly to cGAS, enhancing
73 the sensitivity of cGAS to double-stranded DNA (dsDNA) and its enzymatic activity. Mn²⁺ also
74 enhanced cGAMP-STING binding affinity²⁸. Importantly, Mn²⁺ was a potent innate immune
75 stimulator, inducing type I-IFN and cytokine production in the absence of any infection.

76 Herein we report that Manganese (Mn^{2+}) functions as a universal non-inflammatory adjuvant to
77 induce both humoral and cellular immune responses, particularly CTL activation. A colloidal
78 manganese salt (MnJ) was found to strongly promote dendritic cell maturation and antigen-specific
79 T cell activation. When administrated intranasally, MnJ was also a strong mucosal adjuvant,
80 inducing high levels of IgA antibodies. MnJ showed great adjuvant effects to all tested antigens
81 including inactivated viruses, recombinant proteins and peptides by either intramuscular or
82 intranasal immunization. Thus, we identified the second metal element that functions as adjuvant
83 nearly one hundred years after Alum was found.

84 **Results:**

85 **Mn^{2+} Promotes DC Maturation via cGAS-STING Activation**

86 We previously found that Mn^{2+} is a strong type I-IFN stimulator by activating the cGAS-STING
87 pathway in the absence of any infections *in vitro* and *in vivo*, we thus hypothesized that Mn^{2+}
88 would have some adjuvant potentials. Since APCs play an important role in linking innate and
89 adaptive immunity, we first determined whether Mn^{2+} promotes DC maturation. RNA-seq analysis
90 on Mn^{2+} - or LPS-treated mouse bone marrow derived dendritic cells (BMDCs) revealed that Mn^{2+}
91 induced robust production of both IFN β and various IFN α s, which were not induced by LPS (Fig.
92 1a), together with significantly up-regulated costimulatory molecules CD80 and CD86, mouse
93 MHC-I proteins H-2K/D/Q, immunoproteasome subunits PSMB8/9, peptide transporters TAP1/2
94 and chemokines, including CCL2 and CCL3, that increase the recruitment of immune cells to the
95 injection site. Surprisingly, compared to LPS-treated BMDCs, Mn^{2+} -treated BMDCs did not

96 produce pro-inflammatory cytokines including IL-1 α / β and IL-18, nor IL-10 or IL-12, suggesting
97 that Mn²⁺ triggered a distinct signaling in BMDCs, which were confirmed by qPCR analysis (Fig.
98 1b). Additionally, Mn²⁺-induced expression of costimulatory molecules CD86, CD80 and CD40
99 were lost in BMDCs from *Tmem173*^{-/-}, *Irf3*^{-/-}*Irf7*^{-/-} and *Ifnar*^{-/-} mice, showing that Mn²⁺-induced
100 DC maturation was completely dependent on the activation of the cGAS-STING pathway (Fig.
101 1c).

102 **Mn²⁺ Activates NLRP3 Inflammasome**

103 Alum has been shown to activate the NLRP3 inflammasome and the capacity of Alum to promote
104 antibody production was compromised in *Caspase1*^{-/-}, *Pycard*^{-/-} or *Nlrp3*^{-/-} mice⁹⁻¹¹. To compare
105 the ability of Mn²⁺ and Aluminium-containing adjuvants (Imject® Alum, Alhydrogel® adjuvant
106 and Adju-Phos® adjuvant) to activate innate immunity, we tested the production of type I-IFNs and
107 pro-inflammatory cytokines IL-1 β and IL-18 in peritoneal macrophages treated with Mn²⁺ or
108 various Alums. Only Mn²⁺ induced type I-IFNs (Fig. 2a). Mn²⁺ also activated stronger
109 inflammasome than Imject® Alum and Alhydrogel® Alum did (Fig. 2b, c), which was entirely
110 dependent on ASC, mainly on NLRP3 (Fig. 2d-g). Interestingly, Mn²⁺-induced inflammasome
111 activation in mouse cells was different from THP1 cells, which showed a complete
112 NLRP3-dependence (Fig. 2h, Extended Data Fig. 1a). Moreover, cGAS or STING deficiency did
113 not affect Mn²⁺-activated inflammasome in macrophages from *Cgas*^{-/-}, *Tmem173*^{-/-} mice or
114 *CGAS*^{-/-}, *TMEM173*^{-/-} THP1 cells (Extended Data Fig. 1b-e), indicating that cGAS-STING
115 -induced lysosomal cell death²⁹, or cGAMP production³⁰ was not essential for Mn²⁺-induced

116 inflammasome. Instead, we found that N-acetyl-L-cysteine (NAC, a direct scavenger of ROS),
117 reduced L-glutathione (GSH, an intracellular thiol antioxidant), extracellular K⁺, or 2-APB (a
118 cytosolic Ca²⁺ release inhibitor) all restrained Mn²⁺-induced inflammasome activation (Extended
119 Data Fig. 2a-g). Using a modified culture medium, Hanks' Balanced Salt Solution Deletion of
120 PO₄³⁻ and CO₃²⁻ (herein HBSSD), in which Mn²⁺ and Ca²⁺ did not form particles, we found that
121 Mn²⁺ activated NLRP3 and pyroptosis were essentially independent of particle formation, which is
122 different from Ca²⁺ (Extended Data Fig. 2h, i). mtDNA depletion (Extended Data Fig. 2j) by
123 ethidium bromide²⁸ did not affect Mn²⁺-induced inflammasome activation either (Extended Data
124 Fig. 2k, l). We thus concluded that Mn²⁺ activated stronger NLRP3 inflammasome than Alum did.

125 **Mn²⁺ Activates Inflammasome without IL-1/-18 Production**

126 Consistent with results from RNA-seq and qPCR (Fig. 1a, b), Mn²⁺ treatment did not induce
127 upregulation of *Il1b* and *Il18* in both murine BMDCs and human monocyte derived dendritic cells
128 (Mo-DCs) (Fig. 3a). Interestingly, Alum activated NLRP3 inflammasome in a similar way, neither
129 Mn²⁺ nor Alum induced IL-1/18 production without LPS priming (Fig. 3b, c). The same results
130 were obtained when human peripheral blood mononuclear cells (PBMCs) were treated with Mn²⁺
131 (Fig. 3d). Since clinical studies on various inflammatory diseases suggested the crucial role for
132 IL-1/-18 but not for TNF α , which only amplified and perpetuated the damage³¹, we held that
133 Mn²⁺-activated inflammasome did not cause systematic inflammation but may still be critical for
134 its adjuvant activity (see below).

135 **MnJ Is A Potent Universal Adjuvant**

136 Given that Mn^{2+} induced strong type I-IFN production and NLRP3 inflammasome activation, we
137 reasoned that Mn^{2+} could be used as an adjuvant. To test this, we first immunized C57BL/6 mice
138 with LPS-free chicken ovalbumin protein (OVA) alone or OVA with different Mn^{2+} solutions
139 intramuscularly (i.m.) or intranasally (i.n.) and measured OVA-specific antibodies. Surprisingly,
140 we found that only Mn^{2+} in phosphate buffer saline (PBS), but not Mn^{2+} in normal saline,
141 promoted antibody production (Extended Data Fig. 3a, b). The difference was that Mn^{2+} formed
142 particles in PBS but not in saline, suggesting that soluble Mn^{2+} was unable to induce a local
143 immune response as expected. However, Mn^{2+} particles in PBS tended to aggregate and precipitate
144 with time, thus lost its adjuvant activity (Extended Data Fig. 3c). By screening various manganese
145 compounds, we generated jelly-like Mn^{2+} colloids (MnJ, Mn Jelly) (Extended Data Fig. 3d)
146 consisting of elongate nanoparticles approximately $2 \times 2 \times 6$ nm in size (Extended Data Fig. 3e).
147 MnJ was stable without aggregation in the following experiments. Its adjuvant effect was not
148 weakened after storage at -80 °C for three weeks or longer, or even after freeze–
149 drying/lyophilization (Extended Data Fig. 3f). On the contrary, Alum vaccines are known to be
150 sensitive to freezing and thus hard to store or transport. Interestingly, MnJ triggered comparable
151 inflammasome activation (Extended Data Fig. 3g), but stronger type I-IFN production than $MnCl_2$
152 or Mn^{2+} -PBS did (Extended Data Fig. 3h), probably due to the facilitated transportation into cells
153 as nanoparticles^{32,33}. Compared to $MnCl_2$, which disappeared within hours, MnJ had a much
154 longer muscle retention time up to 8 days at the site of injection, similar to Mn^{2+} -PBS particles
155 (Extended Data Fig. 3i). Splenocytes were isolated from OVA-immunized mice and stimulated
156 with major histocompatibility class II (MHC-II)-binding OVA peptide I-A^b and MHC-I-binding

157 peptide H-2K^b to compare T cell activation. IFN γ induced in splenocytes from OVA-MnJ was
158 higher than OVA-Mn²⁺ (Extended Data Fig. 3j). Consequently, the adjuvant effect of MnJ was
159 significantly better than that of Mn²⁺-PBS (Extended Data Fig. 3a-c).

160 We next evaluated the detailed adjuvant effect of MnJ. MnJ-adjuvanted antibody induction by
161 intramuscularly immunization showed dose-dependence and lasted for at least 6 months (Fig. 4a).

162 Compared to three different Aluminum-containing adjuvants, MnJ induced much stronger
163 OVA-specific IgG1 production and CTL response (Fig. 4b). We also compared MnJ with other
164 adjuvants including the complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA),
165 MF59 and polyetherimide (PEI). We found that MnJ adjuvant effect was even better than CFA (20
166 μ g MnJ vs 50 μ l CFA, i.m.) and MF59 (20 μ g MnJ vs 50 μ l MF59, i.m.) (Extended Data Fig. 3k, l).

167 Also, MnJ boosted specific antibody production against different recombinant protein/peptide
168 antigens, including influenza A/PR8 hemagglutinin A1 peptide, haptenized experimental antigen
169 nitrophenol-conjugatd keyhole limpet hemocyanin (NP-KLH), HBV surface antigen (HBsAg) and
170 HBSS1 fusion protein (containing S (1-223 aa) and PreS1 (21-47 aa))³⁴, and inactivated
171 enterovirus type 71 (EV71) (Fig. 4d). Besides, intranasally immunization revealed that MnJ was
172 also a potent mucosal adjuvant, inducing high levels of IgA antibodies in lung, saliva, and serum
173 for a long time and as good as cholera toxin B (5 μ g MnJ vs 10 μ g CTB, i.n.)³⁵ (Fig. 4e, f).

174 Importantly, compared to CFA-injected mice showing prominent swellings and granulomas with
175 one shoot (Extended Data Fig. 4a), MnJ-injected mice displayed no visible side effects on injection
176 site, body weight, survival or different organs even after repeated administrations (3 shoots in 3

177 consecutive weeks) (Extended Data Fig. 4a-i), suggesting that MnJ is a safe adjuvant with good
178 biocompatibility.

179 **MnJ Promotes Antigen Presentation and T Cell Responses**

180 MF59 and Alum facilitate APCs to engulf antigens and transport them to draining lymph nodes
181 (dLNs), and also induce the differentiation of monocytes to dendritic cells (Mo-DCs) ^{36,37}. So, we
182 next evaluated antigen uptake by APCs and Mo-DC differentiation in dLNs after MnJ
183 administration. We immunized mice in both inguinal regions subcutaneously with fluorescent
184 protein Phycoerythrin (PE) containing MnJ or Alum adjuvant. APCs in inguinal lymph nodes were
185 analyzed by flow cytometry afterwards. The percentage and number of PE-loaded APCs were
186 significantly enhanced 12 and 24 h after MnJ immunization (Fig. 5a). Also, there was a significant
187 increase in the accumulation of Mo-DCs in mice immunized with antigen plus MnJ compared to
188 that in mice immunized with antigen alone (Fig. 5b).

189 The capacity of MnJ to promote BMDC maturation was stronger compared with Mn²⁺, while Alum
190 did not have any effect (Fig. 5c). *In vivo*, MnJ enhanced both CD4⁺ and CD8⁺ T cell proliferation,
191 whereas Alum only induced a weak CD4⁺ T cell proliferation (Fig. 5d). Splenocytes were next
192 isolated from OVA-immunized mice and stimulated with OVA to compare T cell activation. IL-2
193 and IFN γ were highly induced in splenocytes from OVA-MnJ, but not OVA-Alum immunized
194 mice, whereas IL-4 and IL-10 were preferably produced via OVA-Alum immunization (Fig. 5e),
195 indicating that MnJ potently stimulated TH1 response, in addition to TH2 response. Accordingly,
196 *in vivo* cytotoxic assay showed that MnJ immunized mice generated very strong CTL activities in

197 killing OVA-bearing cells, which was absent in Alum immunized mice (Fig. 5f). MnJ also
198 promoted the formation of germinal center (GC) with significantly increased amounts of Tfh and
199 GC B cells (Fig. 5g).

200 **Both cGAS-STING and NLRP3 Inflammasome Contribute to Adjuvant Activity of MnJ**

201 Next, *Tmem173*^{-/-}, *Mavs*^{-/-}, *Nlrp3*^{-/-}, *Nlr4*^{-/-}, *Aim2*^{-/-} or *Pycard*^{-/-} mice were used to test which
202 pathway is involved in MnJ's adjuvant effect. It was found that although *Tmem173*^{-/-}, *Pycard*^{-/-} or
203 *Nlrp3*^{-/-} mice produced diminished OVA-specific antibodies (Extended Data Fig. 5a),
204 *Tmem173*^{-/-}*Pycard*^{-/-} (double knockout, DKO) mice generated extremely decreased OVA-specific
205 antibodies (Fig. 6a-c), suggesting that both cGAS-STING and inflammasome contributed to MnJ
206 adjuvant effect. Further, *Tmem173*^{-/-}*Nlrp3*^{-/-} mice generated a bit more antibodies than
207 *Tmem173*^{-/-}*Pycard*^{-/-} mice did (Fig. 6a-b), indicating the involvement of other ASC-dependent
208 inflammasome activation by MnJ. We also analyzed the expression of *Dock2*, which was reported
209 to be down-regulated in *Pycard*^{-/-} but not *Nlrp3*^{-/-} or *Caspase1*^{-/-} mice³⁸. Quantitative PCR
210 analysis confirmed the same expression of *Dock2* mRNA in WT, *Tmem173*^{-/-}, *Pycard*^{-/-}, *Nlrp3*^{-/-},
211 *Tmem173*^{-/-}*Pycard*^{-/-} and *Tmem173*^{-/-}*Nlrp3*^{-/-} mice (Extended Data Fig. 5b). Consistently,
212 MnJ-promoted germinal center formation was impaired in the DKO (specifically referred to
213 *Tmem173*^{-/-}*Pycard*^{-/-} in the following text) mice (Extended Data Fig. 5c). In addition,
214 MnJ-induced OT-II CD4⁺ T cell proliferation disappeared in the DKO mice (Fig. 6d), along with
215 sharply reduced IFN γ and IL-2 production by peptide-stimulated splenic T cells (Fig. 6e).
216 Consistent with previous results (Fig. 3d, e), no IL-1 β induction was detected in lymph nodes from

217 MnJ-treated mice *in vivo*, despite of ISG production and GSDMD cleavage (Fig. 6f). Since neither
218 Mn^{2+} nor MnJ treatment did induce IL-1 or IL-18 expression *in vitro* or *in vivo*, we reasoned that
219 ASC-mediated inflammasome activation contributed to MnJ adjuvant activity by releasing other
220 DAMPs like uric acid³⁹, but not by IL-1 or TNF α , consistent with previous reports that neither of
221 them was important for adjuvant effect^{22,40}. However, CFA or Monophosphoryl Lipid A (MPLA)
222 induced antibody production did not change much among these mice (Extended Data Fig. 5d, e).

223 **MnJ Is A Potent Adjuvant for Antiviral and Antitumor Vaccines**

224 Next we tested the protection effect of MnJ adjuvanted vaccines against various viruses.
225 Formaldehyde-inactivated Vesicular stomatitis virus (VSV), Herpes simplex virus 1 (HSV-1) and
226 Vaccinia Virus (VACV) were used as vaccines. The optimal dose for each inactivated virus was
227 first determined (VSV and HSV i.m.; VACV i.n.) (Extended Data Fig. 6a-f). MnJ greatly enhanced
228 the protection efficacy of inactivated virus by about 100 times, which is much better than Alum.
229 Consistently, virus titers were decreased by at least 10³ times in MnJ immunized mice (Fig. 7a-f),
230 indicating that MnJ can be applied to all tested virus vaccines through either intramuscular or
231 intranasal immunization, with greatly reduced amounts of inactivated-virus needed for an adequate
232 protection.

233 We particularly tested MnJ in influenza vaccines. Mice immunized with virus protein HA1 (PR8)
234 plus MnJ (i.m. or i.n.) or HA1 plus CTB (i.n.) were completely protected from lethal H1N1
235 A/PR8/34 virus challenge, while Alum adjuvant only showed mild protection (Fig. 7g, h, Extended
236 Data Fig. 6g-i). Importantly, MnJ enhanced the protection effect of inactivated PR8 vaccine by at

237 least 10 times (Fig. 7i). Moreover, MnJ exhibited superior protection against heterologous
238 influenza viruses to mice immunized with MnJ-adjuvanted inactivated PR8 or HA1 protein,
239 followed by lethal H1N1 WSN or H3N2 challenge (Extended Data Fig. 6j).

240 Finally, the adjuvant effect of MnJ on cancer vaccines was evaluated. Mice were immunized three
241 times before inoculated with melanoma cell B16-OVA subcutaneously. Tumor growth was greatly
242 suppressed in OVA-MnJ, but not OVA-Alum, immunized mice (Fig. 7j, Extended Data Fig. 7a), in
243 line with prominently improved survival (Fig 7k) and increased tumor-infiltrating CD4⁺ and CD8⁺
244 T cells (Extended Data Fig. 7b, c), confirming CTL inducing activity of MnJ. In pulmonary
245 metastasis model, OVA-MnJ immunization greatly blocked lung metastases in the WT, but not
246 *Tmem173^{-/-}Pycard^{+/-}* mice (Fig. 7l, m, Extended Data Fig. 7d), which is consistent with the
247 proliferation of CD8⁺ OT-I T cells (Extended Data Fig. 7e). In addition, OVA-specific antibody
248 production and CD8⁺ T cell activation analyzed by tetramer assays were only detected in
249 MnJ-OVA immunized WT mice (Fig. 7n, Extended Data Fig. 7f, g), suggesting its potentials in
250 tumor therapies.

251 **DISCUSSION**

252 The adjuvant activity of Aluminum was found by Alexander Glenny and his colleagues in 1926⁴¹,
253 now almost one century later, we reported the adjuvant activity of another metal-Manganese.
254 Adjuvant effect is essentially attributed by type I-IFN-promoted dendritic cell maturation and
255 migration to prime adaptive immune responses along with 1) local up-regulation of chemokines,
256 including CCL2 (MCP-1) and CCL3 (MIP-1 α) to recruit immune cells to the injection site; 2)

257 increase of antigen uptake by immune cells; 3) induction of monocyte differentiation into dendritic
258 cells ^{8,36,37}. We found that Mn²⁺ induced APCs to produce both IFN β and various IFN α s, which
259 were surprisingly not induced by LPS, together with many co-stimulatory and MHC molecules
260 crucial for antigen presentation and chemokines for immune cell recruitment. MnJ also strongly
261 enhanced antigen uptake by APCs and the differentiation of monocytes to dendritic cells.
262 Importantly, Mn²⁺ did not induce the production of pro-inflammatory cytokines IL-1 and IL-18 in
263 human or mouse *in vitro* and *in vivo*. Therefore, MnJ demonstrated superior adjuvant effects as it
264 induces humoral, cellular, and mucosal immune responses, particularly CTL activation, without
265 detected side-effects. In addition, the adjuvant effect of MnJ was stable even after repeated
266 freeze-drying cycles, whereas Alum is sensitive to freezing and requires cold-chain temperature
267 control ^{42,43}. MnJ showed great adjuvant effects to all tested vaccine antigens including inactivated
268 viruses, recombinant protein subunits and peptides, thus it can significantly reduce the amount of
269 viruses needed. Particularly, its tumor antigen-specific CTL activity by either intramuscular or
270 intranasal MnJ immunization indicated a great potential for cancer vaccines. Based on these results,
271 we believe that MnJ has great potentials for the development of potent but safe vaccines. The
272 component simplicity and steadiness of MnJ, the low cost and wide availability of Mn make this
273 adjuvant even more promising.

274 Although Alum adjuvanted vaccines have been proven to be safe in most cases, some studies
275 showed that aluminum accumulation are associated with long-lasting macrophagic myofasciitis
276 (MMF) ⁴⁴, nervous disorders ⁴⁵ and bone disease ⁴⁶ in some patients. The FDA-approved doses of
277 850 μ g, 1140 μ g, and 1250 μ g Alum per vaccine were determined according to antigenicity and

278 effectiveness of vaccine, not including safety consideration⁴⁷⁻⁴⁹. After vaccine injection, Alum has
279 been found in the injected muscle, draining lymph nodes and spleen 9 months in humans⁵⁰ or even
280 12 years in patients with ASIA (Autoimmune/inflammatory syndrome induced by adjuvants)^{51,52}.
281 Consistently, we found that intramuscularly injected Alum in mice did not show obvious clearance
282 2 weeks after injection whereas injected MnJ was beyond detection after 8 days. Importantly, MnJ,
283 but not Alum, could be readily decomposed into free Mn²⁺ ions by many common acidic metabolic
284 products or under acidic environment (data not shown). In addition, tumor microenvironment is
285 characterized by anomalous metabolic properties and acidic environment⁵³, which might be
286 beneficial for the application of MnJ in antitumor therapy. Excessive Mn accumulation in the
287 central nervous system causes neurologic toxicity in occupational cohorts through inhalation from
288 welding or smelting²⁶. However, we found that 20 µg MnJ induced higher antibody production
289 than 1444 µg Imject® Alum, 1444 µg Alhydrogel® adjuvant 2%, 2259µg Adju-Phos® adjuvant
290 (500 µg Alum in each) did, indicating MnJ is at least 25 times more potent than Alum in terms of
291 inducing humoral immune response, which means that much smaller amount of MnJ can achieve
292 the same effect.

293 There is still controversy about the function of NLRP3 inflammasome on adjuvant effect of
294 aluminum salt¹². These different conclusions may result from different aluminum salts or mice
295 backgrounds used. There is also another view that ASC has an inflammasome-independent role in
296 shaping adaptive immunity, for regulating the expression of Dock2, which is important for antigen
297 uptake and lymphocyte mobility³⁸. However, we found that STING, NLRP3 or ASC deficiency
298 did not affect the expression of Dock2 in macrophages or lymphocytes. Interestingly, manganese

299 salts administration did not upregulate pro-IL-1 β or pro-IL-18 production, which means that MnJ
300 activate adaptive immune responses partly through an inflammasome-dependent but inflammatory
301 cytokines-independent manner. In this regard, there may be some other stimulators upregulated by
302 MnJ and released by pyroptotic death of cells. However, even though we did not detect IL-1/-18
303 production by macrophages or PBMCs treated with MnCl₂ alone, these cells may generate these
304 cytokines when treated with inactivated pathogens plus MnCl₂. IL-1 and IL-18 can promote the
305 infiltration of neutrophils and enhance immune responses after immunization with adjuvants like
306 Alum or ISCOMATRIX^{40,54,55}.

307 In addition, similar to Alum adjuvant, the MnJ nanoparticles were also able to absorb antigens
308 like OVA, GFP or PE proteins (data not shown). The physical depot effect of MnJ retained antigens
309 at the injection site and enhanced the uptake of antigens by APCs. Generally, MnJ is an adjuvant
310 owning the properties of both immune potentiator and delivery system. Because of its excellent
311 adjuvant activities and stability against repeated freezing-thaw treatment, Mn-based adjuvants
312 would be especially useful in veterinary vaccines with the following three additional advantages. 1)
313 MnJ showed a very nice dose-dependent adjuvant activity intramuscularly or intranasally, with 20
314 μ g MnJ per mouse showing stronger antibody inducing activity than any tested adjuvants including
315 CFA; 2) High MnJ dose (up to several mg/shot) and repeated administrations to mice, rabbits or
316 pigs (data not shown) did not cause any visible damage or inflammation such as swellings and
317 granulomas at the injection sites or in various organs; 3) Mammals keep tissue Mn levels via tight
318 control of both absorption and excretion, as normally only 1–5% of ingested Mn is absorbed into
319 the body⁵⁶ and excessive dietary Mn causes reduced Mn absorption and enhanced Mn metabolism

320 and excretion⁵⁷⁻⁵⁹. Therefore, even highly elevated Mn levels in meats caused by Mn-containing
321 veterinary vaccines would unlikely increase gastrointestinal Mn absorption by humans. In fact, Mn
322 contents in whole grains, rice, and nuts are around or more than 30 mg Mn/kg or even 110-140 mg
323 Mn/kg in wheat bran, much higher than those in mammals that are between 0.3 and 2.9 mg Mn/kg
324 wet tissue weight, confirming the tight Mn absorption by animals.

325 **References and Notes:**

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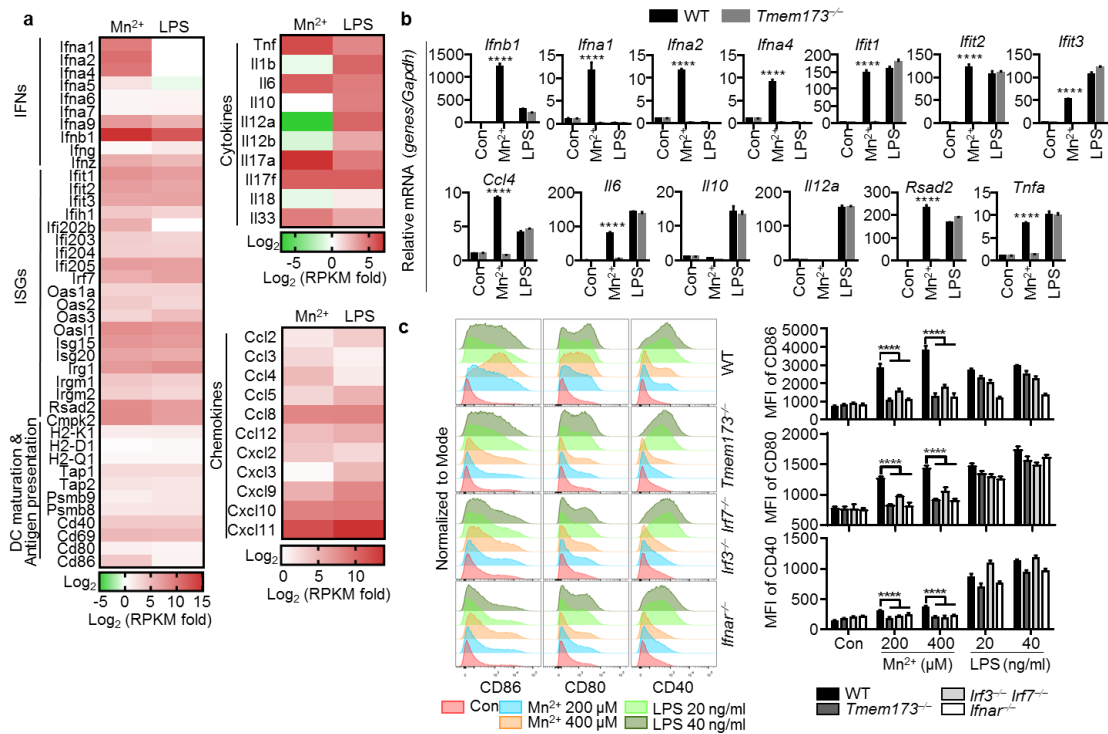
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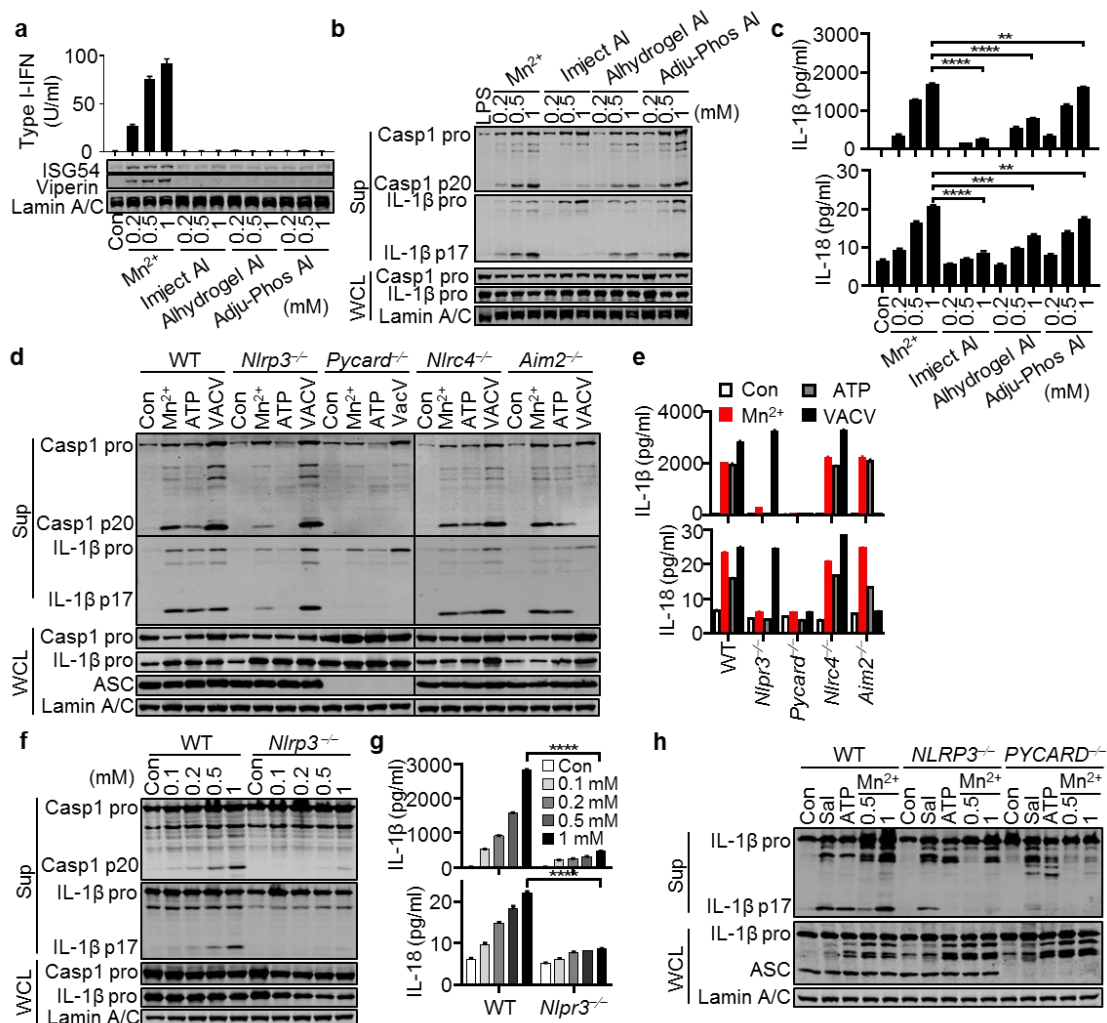
497 **Data and materials availability:** All data supporting the findings of this study are available within
498 the paper and its supplementary materials.

499 RNA-seq data have been deposited in Gene Expression Omnibus under accession no. GSE126586.



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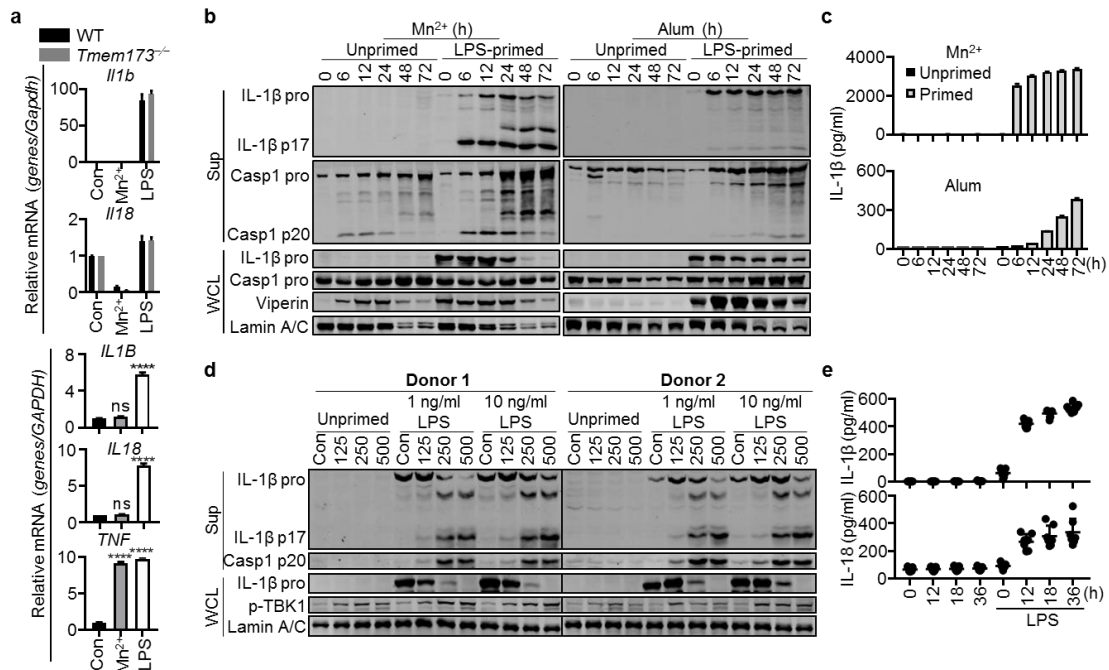
501 **Fig. 1| Mn²⁺ Promotes DC Maturation via cGAS-STING Activation. a**, Heatmap of RNA-seq
 502 analysis. BMDCs were untreated or treated with MnCl₂ (200 μM) or LPS (100 ng/ml) for 20 h.
 503 Heatmap was made by calculating log₂ ((treated RPKM)/(control RPKM)). **b**, Quantitative
 504 RT-PCR analysis of the indicated gene expression in the WT and *Tmem173*^{-/-} BMDCs treated with
 505 MnCl₂ (200 μM) or LPS (100 ng/ml) for 20 h. **c**, BMDCs from the WT, *Tmem173*^{-/-}, *Irf3*^{-/-}*Irf7*^{-/-}
 506 or *Ifnar*^{-/-} mice were treated with the indicated concentrations of MnCl₂ or LPS for 20 h. CD86,
 507 CD80 and CD40 expression was analyzed by FACS. One representative experiment of at least
 508 three independent experiments is shown, and each was done in triplicate. Error bars represent SEM;
 509 **b**, data were analyzed by two-way ANOVA; **c**, data were analyzed by an unpaired t test. ns, not
 510 significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.



511

512 **Fig. 2| Mn²⁺ Activates NLRP3 Inflammasome. a,** Western blot (lower) and Type I-IFN
 513 production analysis (upper) of mouse peritoneal macrophages treated with the indicated
 514 concentrations of MnCl₂ or Aluminium salts (Imject® Alum, Alhydrogel® adjuvant 2%,
 515 Adju-Phos® adjuvant) for 18 h. **b, c,** Western blot (**b**) and ELISA analysis (**c**) of inflammasome
 516 activation of LPS-primed C57BL/6 peritoneal macrophages treated with the indicated
 517 concentrations of MnCl₂ or Aluminium salts for 5 h. Supernatants (Sup) and whole cell lysates
 518 (WCL) were analyzed by immunoblotting with the indicated antibodies. **d, e,** Western blot (**d**) and
 519 ELISA analysis (**e**) of inflammasome activation of LPS-primed WT, *Nlrp3*^{-/-}, *Pycard*^{-/-}, *Nlrp4*^{-/-}

520 and *Aim2*^{-/-} peritoneal macrophages treated with MnCl₂ (0.5 mM), ATP (5 mM) or VACV (MOI =
521 10). **f, g**, Western blot (**f**) and ELISA analysis (**g**) of inflammasome activation of LPS-primed WT
522 and *Nlrp3*^{-/-} peritoneal macrophages treated with the indicated concentrations of MnCl₂ for 5 h. **h**,
523 Western blot analysis of inflammasome activation of LPS-primed THP1 cells treated with
524 Salmonella (Sal, MOI = 10), ATP (5 mM) or MnCl₂ (0.5 and 1 mM). One representative
525 experiment of at least three independent experiments is shown, and each was done in triplicate.
526 Error bars represent SEM; **a, c, g**, data were analyzed by an unpaired t test. ns, not significant; * P
527 < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.



528

529 **Fig. 3| Mn²⁺ Activates Inflammasome without IL-1/18 Production.** **a**, Quantitative RT-PCR

530 analysis of the indicated gene expression in the WT and *Tmem173*^{-/-} BMDCs (upper) or human

531 Mo-DCs (lower) treated with MnCl₂ (200 μM) or LPS (100 ng/ml) for 20 h. **b**, **c**, Unprimed or

532 LPS-primed mouse peritoneal macrophages were treated with MnCl₂ (200 μM) or Alum (100

533 μg/ml) for the indicated times. Supernatants (Sup) and whole cell lysates (WCL) were analyzed by

534 immunoblotting with the indicated antibodies (**b**). IL-1β in supernatants was analyzed by ELISA

535 (**c**). **d**, **e**, Unprimed or LPS (1 ng/ml or 10 ng/ml)-primed human PBMCs were treated with MnCl₂

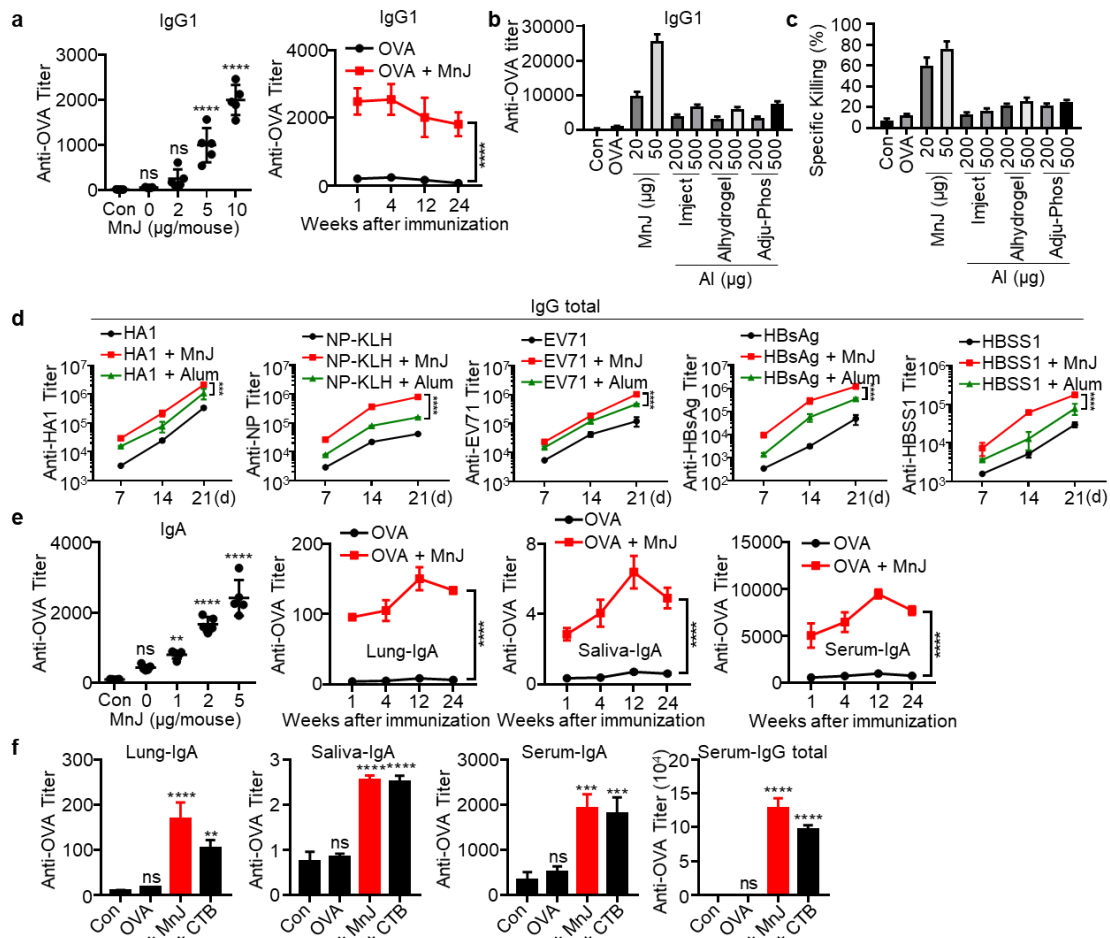
536 (200 μM) for the indicated times. Supernatants and whole cell lysates were analyzed by

537 immunoblotting with the indicated antibodies (**d**). Human IL-1β and IL-18 in supernatants were

538 analyzed by ELISA (**e**). One representative experiment of at least three independent experiments is

539 shown, and each was done in triplicate. Error bars represent SEM; **a**, data were analyzed by an

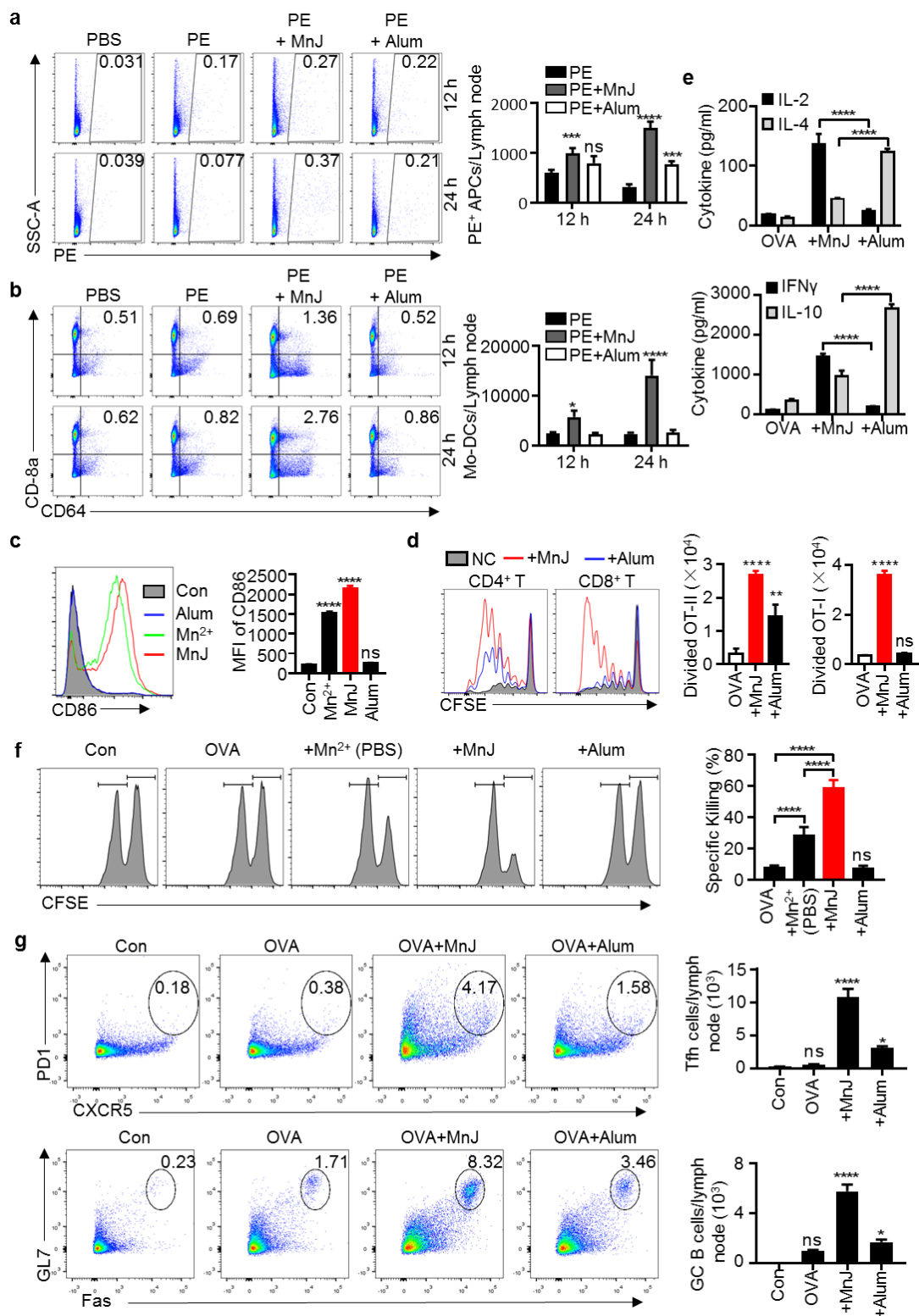
540 unpaired t test. ns, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.



541

542 **Fig. 4| MnJ Is A Potent Universal Adjuvant.** **a**, The WT mice (C57BL/6) were immunized
 543 intramuscularly with PBS or OVA (10 µg) + MnJ (0, 2, 5 or 10 µg) on day 0, 7 and 14. Sera were
 544 collected on day 21 to measure OVA-specific IgG1 by ELISA (left, n = 5). Time course of
 545 OVA-specific IgG1 in sera from mice immunized intramuscularly with OVA (10 µg) + MnJ (10 µg)
 546 for three times (right, n = 3). **b**, **c**, The WT mice were immunized intramuscularly with PBS, OVA
 547 (10 µg), OVA (10 µg) + MnJ (5 µg) or OVA (10 µg) + indicated amounts of Aluminium salts. Sera
 548 were collected on day 21 to measure OVA-specific IgG1 by ELISA (**b**) (n = 4). OVA-specific
 549 cytotoxicity was measured on day 21 in an *in vivo* killing assay (**c**) (immunized on day 0, 7 and 14,
 550 n = 4). **d**, The WT mice were immunized intramuscularly with the indicated antigen (5 µg), antigen

551 (5 μ g) + MnJ (10 μ g) or antigen (5 μ g) + Alum (1320 μ g) on day 0, 7 and 14. Sera were collected
552 on day 7, 14 and 21 to measure HA1, NP, EV71, HBsAg or HBSS1-specific IgG total (n = 3). **e**,
553 The WT mice were immunized intranasally with PBS or OVA (10 μ g) + MnJ (0, 1, 2 or 5 μ g) on
554 day 0, 7 and 14. Sera were collected on day 21 to measure OVA-specific IgA by ELISA (left, n =
555 5). Time course of OVA-specific IgA in BALF, saliva and serum as indicated from mice
556 immunized intranasally with OVA (10 μ g) + MnJ (5 μ g) for three times (n = 3). **f**, OVA-specific
557 IgA and IgG total were measured by ELISA on day 21 after immunization with OVA (10 μ g), OVA
558 (10 μ g) + MnJ (5 μ g) or OVA (10 μ g) + CTB (10 μ g) intranasally on day 0, 7 and 14 (n = 3). One
559 representative experiment of at least three independent experiments is shown, and each was done
560 in triplicate. Error bars represent SEM; **d**, data were analyzed by two-way ANOVA; **a**, **e**, **f**, data
561 were analyzed by an unpaired t test. ns, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001;
562 **** P < 0.0001.

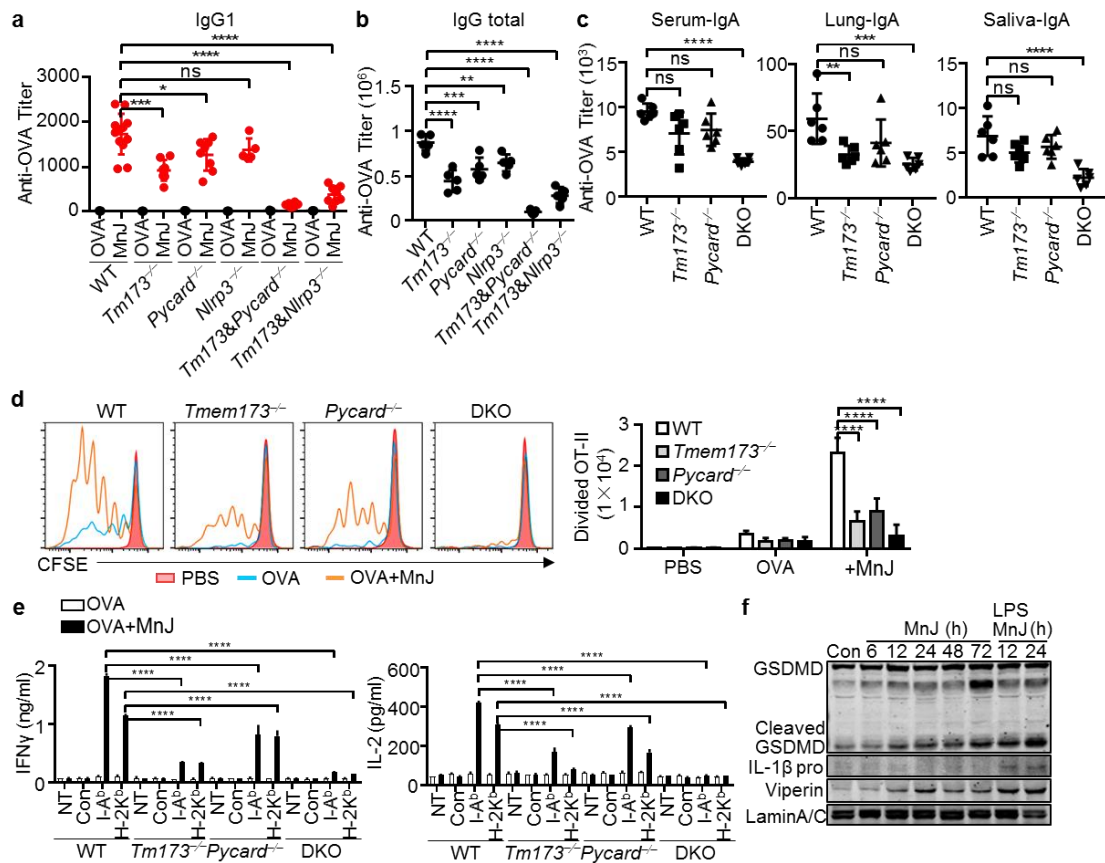


563

564 **Fig. 5| MnJ Promotes Antigen Presentation and T Cell Responses.** a, b, The WT mice were

565 immunized subcutaneously with PBS, Phycoerythrin (PE, 10 μg), PE (10 μg) + MnJ (20 μg) or PE

566 (10 µg) + Alum (200 µg). Inguinal lymph nodes were collected 12 or 24 h later (n = 3). Ratio and
567 number of PE⁺ APCs (**a**) and Mo-DCs (**b**) in dLN cells were analyzed by FACS. Live cells were
568 identified by DAPI staining. Among live singlet cells, APCs were identified as the cell subset
569 single or double positive for CD11c and F4/80. Among APCs, Mo-DCs were identified as CD8a⁺
570 CD64⁺ cells. **c**, BMDCs were treated with MnCl₂ (20 µg/ml), MnJ (20 µg/ml) or Alum (20 µg/ml)
571 for 20 h. CD86 expression was analyzed by FACS. **d**, CD45.1⁺ OT-I CD8⁺ or OT-II CD4⁺ T cells
572 were labeled with CFSE and transferred to CD45.2⁺ WT mice. These mice were then immunized
573 with OVA (1 µg), OVA (1 µg) + MnJ (10 µg) or OVA (1 µg) + Alum (1320 µg). After 3 days, T cell
574 proliferation was analyzed by FACS (n = 3). **e**, The WT mice were immunized intramuscularly
575 with OVA (10 µg), OVA (10 µg) + MnJ (10 µg) or OVA (10 µg) + Alum (1320 µg) on day 0, 7 and
576 14. Splenocytes were collected on day 21, and stimulated with OVA (100 µg/ml). IL-2, IL-4, IFN γ
577 and IL-10 secreted by T cells were measured by ELISA (n = 3). **f**, OVA-specific cytotoxicity was
578 measured on day 21 in an *in vivo* killing assay (immunized on day 0, 7 and 14, n = 4). **g**, Numbers
579 of Tfh or GC B cells in dLN from WT mice were analyzed by FACS. Live cells were identified by
580 DAPI staining. Among live singlet cells, CD4⁺ T cells were identified as the cell subset double
581 positive for CD3 and CD4. Among CD4⁺ T cells, Tfh cells were identified as PD1⁺ CXCR5⁺ cells.
582 B cells were identified as the cell subset double positive for CD45 and B220. Among B cells, GC B
583 cells were identified as Fas⁺ GL7⁺ cells. One representative experiment of at least three
584 independent experiments is shown, and each was done in triplicate. Error bars represent SEM; **a, b,**
585 **e**, data were analyzed by two-way ANOVA; **c, d, f, g**, data were analyzed by an unpaired t test. ns,
586 not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.



587

588 **Fig. 6| Both cGAS-STING and NLRP3 Inflammasome Contribute to Adjuvant Activity of**

589 **MnJ. a, b**, OVA-specific IgG1 (a) and IgG total (b) from the WT, *Tmem173*^{+/-}, *Pycard*^{+/-}, *Nlrp3*^{+/-},

590 *Tmem173*^{+/-}*Pycard*^{+/-} and *Tmem173*^{+/-}*Nlrp3*^{+/-} mice were measured by ELISA on day 14 after

591 immunization with OVA (10 μ g) or OVA (10 μ g) + MnJ (10 μ g) intramuscularly on day 0 and 7 (n >

592 5). c, OVA-specific IgA from the WT, *Tmem173*^{+/-}, *Pycard*^{+/-} and *Tmem173*^{+/-}*Pycard*^{+/-} DKO mice

593 was measured by ELISA on day 21 after immunization with OVA (10 μ g) or OVA (10 μ g) + MnJ

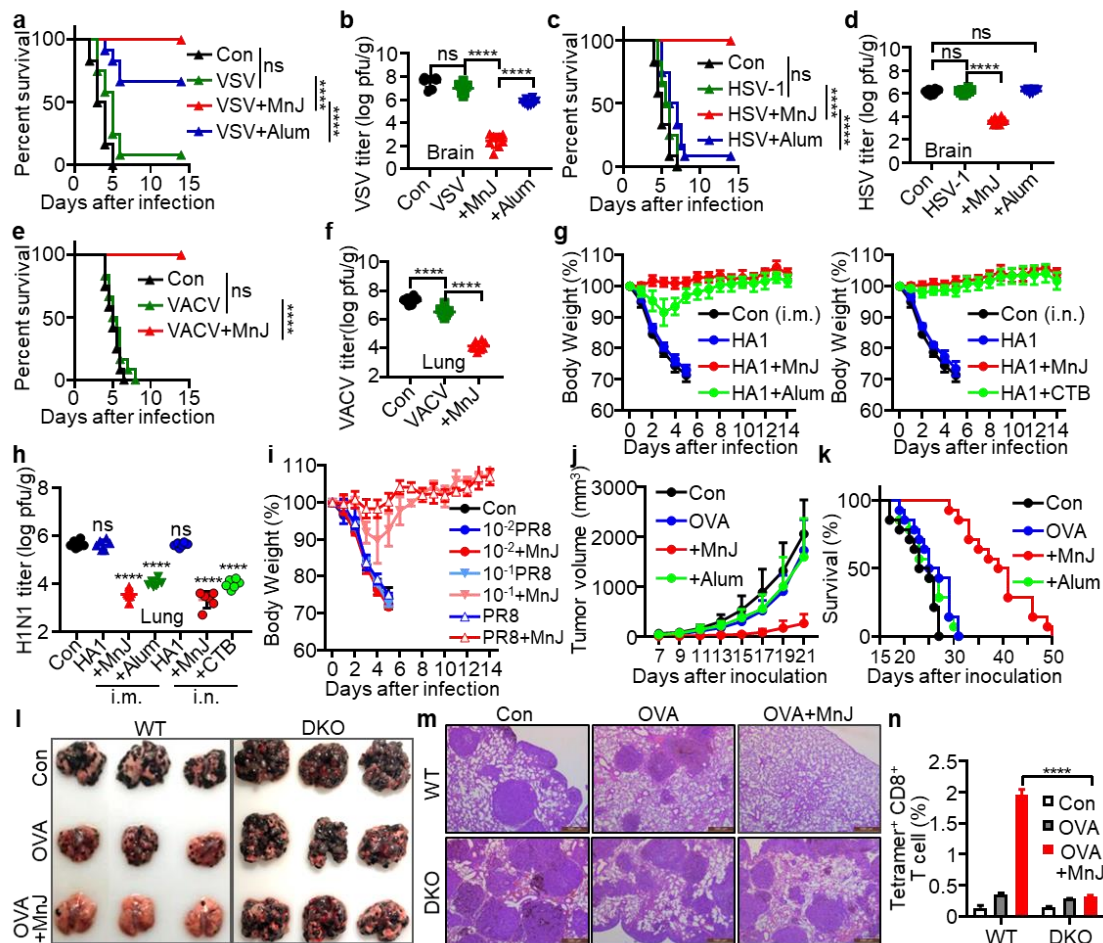
594 (5 μ g) intranasally on day 0, 7 and 14 (n = 6). d, CD45.1⁺ OT-II CD4⁺ T cells were labeled with

595 CFSE and transferred to CD45.2⁺ WT, *Tmem173*^{+/-}, *Pycard*^{+/-} and *Tmem173*^{+/-}*Pycard*^{+/-} DKO mice.

596 These mice were then immunized with PBS, OVA (1 μ g) or OVA (1 μ g) + MnJ (10 μ g). After 3

597 days, T cell proliferation was analyzed by FACS (n = 3). e, The WT, *Tmem173*^{+/-}, *Pycard*^{+/-} and

598 *Tmem173^{-/-}Pycard^{-/-}* DKO mice were immunized as (a). Splenocytes were collected on day 21,
599 and stimulated with OVA peptides. IFN γ and IL-2 secreted by T cells were measured by ELISA (n
600 = 3). f, The WT mice were immunized with MnJ (100 μ g) or LPS (20 μ g) + MnJ (100 μ g)
601 intramuscularly for the indicated times. Lysates of draining lymph node cells were analyzed by
602 immunoblotting with the indicated antibodies. One representative experiment of at least three
603 independent experiments is shown, and each was done in triplicate. Error bars represent SEM; data
604 were analyzed by an unpaired t test. ns, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; ****
605 P < 0.0001.



606

607 **Fig. 7| MnJ Is A Potent Adjuvant for Antiviral and Antitumor Vaccines. a, b,** The WT mice
 608 were immunized intramuscularly with PBS, inactivated VSV (10^5 pfu), inactivated VSV (10^5 pfu)
 609 + MnJ (10 μ g) or inactivated VSV (10^5 pfu) + Alum (1320 μ g) on day 0. On day 10, these mice
 610 were infected intravenously with a lethal dose of VSV. The survival was monitored for 2 weeks (n
 611 = 12) (**a**). Viral loads in brain were measured 5 days after infection (n = 8) (**b**). **c, d,** The WT mice
 612 were immunized intramuscularly with PBS, inactivated HSV-1 (10^3 pfu), inactivated HSV-1 (10^3
 613 pfu) + MnJ (10 μ g) or inactivated HSV-1 (10^3 pfu) + Alum (1320 μ g) on day 0. On day 10, these
 614 mice were infected intraperitoneally with a lethal dose of HSV-1. The survival was monitored for 2
 615 weeks (n = 12) (**c**). Viral loads in brain were measured 5 days after infection (n = 8) (**d**). **e, f,** The

616 WT mice were immunized intranasally with PBS, inactivated VACV (2×10^4 pfu) or inactivated
617 VACV (2×10^4 pfu) + MnJ (5 μ g) on day 0 and 7. On day 14, these mice were infected intranasally
618 with a lethal dose of VACV. The survival was monitored for 2 weeks (n = 12) (e). Viral loads in
619 lung were measured 5 days after infection (n = 8) (f). **g**, The WT mice were immunized
620 intramuscularly (i.m.) with HA1 (5 μ g), HA1 (5 μ g) + MnJ (10 μ g), HA1 (5 μ g) + Alum (1320 μ g)
621 or intranasally (i.n.) with HA1 (5 μ g), HA1 (5 μ g) + MnJ (10 μ g), or HA1 (5 μ g) + CTB (10 μ g) on
622 day 0, 7 and 14. On day 21, these mice were infected intranasally with a lethal dose of PR8. The
623 survival was monitored for 2 weeks (n = 10). **h**, The WT mice were immunized intranasally with
624 PBS, inactivated PR8 (5×10^6 pfu), 10^{-1} PR8 (5×10^5 pfu) or 10^{-2} PR8 (5×10^4 pfu) with or
625 without MnJ (5 μ g) on day 0 and 7. On day 14, these mice were infected intranasally with a lethal
626 dose of PR8. The body weight was recorded for 2 weeks (n = 3). **i**, Viral loads in lungs from mice
627 in (g) were measured 5 days after infection (n = 6). **j**, **k**, The WT mice were immunized
628 intramuscularly with PBS, OVA (10 μ g), OVA (10 μ g) + MnJ (20 μ g) or OVA (10 μ g) + Alum
629 (1320 μ g) on day 0, 7 and 14. On day 21, these mice were inoculated with B16-OVA-Fluc cells (3
630 $\times 10^5$) subcutaneously. Tumor volume (**j**) was measured and survival (**k**) was monitored (n = 14). **l**,
631 **m**, **n**, The WT and DKO mice were immunized intramuscularly with PBS, OVA (10 μ g), or OVA
632 (10 μ g) + MnJ (20 μ g) on day 0, 7 and 14. On day 21, these mice were inoculated with
633 B16-F10-OVA (3×10^5) intravenously. Images (**l**) and HE staining (**m**) of lung tissues were
634 recorded 20 days after inoculation (n = 6). The percentage of tetramer⁺ CD8⁺ T cells in spleens of
635 these mice was analyzed by FACS on day 21 (n = 3) (**n**). One representative experiment of at least
636 three independent experiments is shown, and each was done in triplicate. Error bars represent SEM;

637 **b, d, f, h, n**, data were analyzed by an unpaired t test; **a, c, e, k**, survival plot data were analyzed

638 with log-rank (Mantel–Cox) tests. ns, not significant; * P < 0.05; ** P < 0.01; *** P<0.001; **** P

639 < 0.0001.