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31 Abstract

32 Lung localized CD4 T cells play a critical role in the control of influenza virus infection,
33 acting synergistically along with other immune cell subsets, and mediating direct effector
34 functions within the lung. However, current influenza vaccination strategies are predominantly
35 intended to elicit serum antibody responses, and this approach sometimes fails to provide
36 protection. We explored the use of an intranasal vaccination regimen targeting the highly
37 conserved influenza nucleocapsid protein to elicit lung localized CD4 T cell responses. The
38 platform consists of a non-toxic self-assembling nanolipoprotein (NLP) that is stable under
39 biological conditions and can bind diverse antigens and adjuvant compounds. By targeting
40 nanolipoprotein:nucleocapsid protein (NLP:NP) complexes to the lung, we find that this
41 immunization regimen elicits a polyfunctional subset of lung-localized CD4 T cells. A subset of
42 these lung CD4 T cells localize to the airway, where they can act as early responders following
43 encounter with cognate antigen. Polyfunctional CD4 cells isolated from airway and lung
44 parenchyma produce significantly more effector cytokines IFN γ and TNF α , as well as more
45 frequent degranulation relative to cells isolated from secondary lymphoid organs. These NP-
46 specific CD4 T cells were found to persist long-term post vaccination. Intranasal NLP:NP also
47 potentiates mucosal IgG and IgA responses. These results highlight the potential of NLPs and
48 intranasal vaccination to enhance durable lung-localized immune responses that can persist at the
49 site of influenza infection.

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54 **Author summary**

55 Influenza virus infection poses a world-wide health risk, especially in the case of an
56 emergent pandemic virus. Exploiting new, more efficacious strategies to potentiate influenza-
57 specific immune responses is important, particularly for at risk populations. Next generation
58 influenza vaccines will need to carefully consider the antigenic formulation and route of
59 administration to more fully engage the humoral and cellular branches of the immune system. A
60 unique subset of the cellular immune response, known as tissue resident T cells, may serve as a
61 promising target for rational vaccine development efforts. Tissue resident T cells are localized to
62 the site of infection where they can rapidly respond to infection. By targeting conserved epitopes,
63 tissue resident T cells can respond to infection by diverse viral isolates due to the broad antigen-
64 specificity of the polyclonal T cell response. In this study, we evaluated the endogenous antigen-
65 specific CD4 T cell response to intranasal vaccination with a highly-conserved influenza antigen
66 conjugated to a novel vaccine platform. The route of administration and co-delivery of vaccine
67 components on the vaccine platform can impact the CD4 T cell response to immunization, in
68 terms of the magnitude and localization of antigen-specific cells. Vaccination with the assembled
69 vaccine platform by the intranasal route localizes a population of CD4 T cells with robust
70 effector functions to the airways and lung tissue, sites where this effector function is most
71 important.

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74 as part of the manuscript to immediately follow the abstract. This text is subject to editorial change,
75 should be written in the first-person voice, and should be distinct from the scientific abstract. Aim to
76 highlight where your work fits within a broader context; present the significance or possible implications of
77 your work simply and objectively; and avoid the use of acronyms and complex terminology wherever
78 possible. The goal is to make your findings accessible to a wide audience that includes both scientists
79 and non-scientists. Authors may benefit from consulting with a science writer or press officer to ensure
80 they effectively communicate their findings to a general audience.]

81 **Introduction**

82 Disease outbreaks caused by influenza A viruses result in considerable human morbidity,
83 mortality, and economic burden each year (1-4). Currently, intramuscular vaccination is the
84 primary clinical strategy employed to combat influenza infection (5-7). Although traditional
85 inactivated split influenza virus vaccines have been successful in alleviating disease prevalence
86 and severity, this strategy sometimes fails to elicit sufficiently protective responses (8-11).
87 Failure to elicit protective responses may be due to antigenic mismatch between circulating and
88 vaccine seed viruses, emergence of novel viruses which arise from antigenic shift or drift, or
89 mutations which occurred during vaccine production in eggs (12, 13). Even in cases where
90 sterilizing antibody responses are successfully generated against hemagglutinin (HA), antibodies
91 to the immunodominant head region of HA typically predominate. This confers less protection
92 against heterosubtypic or variant strains of influenza infection than responses directed against the
93 more conserved stalk region (14, 15). While many individuals have antibodies directed against
94 the stalk region, viral escape mutants have been shown to emerge in the presence of antibodies
95 directed against either the immunodominant head or stalk region, suggesting that HA is a
96 constantly moving antigenic target (16). Further, anti-HA antibody titers in repeatedly vaccinated
97 subjects can begin to decline within 6 months of vaccination (17). To overcome the limitations of
98 current subunit influenza vaccination strategies, development of universal influenza vaccines that
99 elicit improved durability and breadth of immune protection has become a high priority in the
100 field (18).

101 To achieve the goal of universal influenza vaccines that induce broadly protective
102 immunity, it would be advantageous to develop strategies that more fully induce and harness
103 both lung-localized and T cell mediated immunity. Included in these efforts is a focus on the

104 immune response to influenza antigens that are broadly conserved among virus strains and to
105 explore new routes of delivery (18). Numerous studies have implicated memory T cells as key
106 mediators of the adaptive immune response to influenza infections, especially responses to
107 heterosubtypic influenza infections (19-22). CD4 and CD8 T cells play critical, but distinct roles
108 in mediating protection from viral infection. CD4 T cells are distinguished from CD8 T cells in
109 the diversity of protective mechanisms that they contribute to immunity. These include anti-viral
110 cytokine production, enhanced recruitment of innate cells into the respiratory tract, direct
111 cytotoxicity, providing help to naïve CD8 T cells and for B cell production of high affinity, HA-
112 specific B cells that can promote a sterilizing antibody response (23-26). As well as a high
113 degree of functional diversity, the memory CD4 T cell pool is characterized by heterogeneity in
114 localization, persistence, and antigen specificity (27, 28). The highly diverse antigen specificity
115 of memory T cells contributes to heterosubtypic immunity by targeting highly conserved
116 epitopes within HA, NP, polymerase, and matrix viral proteins (12, 29).

117 Vaccines designed to elicit lung-localized T cell immunity will need to carefully consider
118 the route of vaccine administration and vaccine formulation, as these factors are critical in
119 determining the position and function of these T cells. In contrast to other tissues, local antigen
120 recognition within the lung is required to elicit stable T cell populations in the lung (12, 13, 30).
121 Studies of infection and vaccination suggest that CD4 T cells primed via an intranasal route
122 display persistent retention in the lung tissue, and enhanced protective potential relative to CD4
123 T cells isolated from secondary lymphoid organs (31-33). Inclusion of immunostimulatory
124 adjuvants in vaccine formulations can increase durability and magnitude of vaccine responses,
125 affect T cell repertoire clonal diversity, and influence polarization of responding T cells (34-40).
126 Additionally, co-localization of antigen and adjuvant can enable enhanced responses with

127 sparing doses of antigen and adjuvant relative to soluble formulations (41-43). Thus, a rational
128 vaccination approach that employs co-delivery of conserved viral antigen and Th1 polarizing
129 adjuvant by an intranasal route may elicit more broadly protective immunity by potentiating CD4
130 T cells that reside in, or can be rapidly recalled to, the site of infection to directly mediate
131 effector function and synergize with other immune cell subsets.

132 While much is known about CD8 T cell and antibody responses to vaccination, the CD4
133 T cell response remains comparatively understudied. To better understand the localization,
134 phenotype, and persistence of CD4 T cells elicited by intranasal vaccination, we developed and
135 tested a biocompatible nanolipoprotein platform conjugated to the highly conserved influenza
136 antigen NP. Previous influenza vaccine design efforts have been hindered by requirements for
137 high doses (44), priming of pathogenic T cell responses (45), or instability in biological
138 conditions (46). In this report, we characterize the endogenous polyclonal CD4 T cell response to
139 this novel self-assembling vaccine platform, which has the potential to capture and present
140 diverse arrays of antigens and adjuvants while remaining stable under biological conditions (41,
141 47-49). Using this platform to study lung-resident CD4 T cell populations, we evaluated the
142 functional potential, homing, and persistence of CD4 T cells elicited by this vaccine platform and
143 how they can contribute to our understanding of tissue-localized CD4 T cells providing
144 protective immunity against influenza.

145 **Results**

146 **Nanolipoproteins self-assemble into stable complexes of antigen and adjuvant**

147 In order to better utilize animal models for vaccine studies, we considered the well-
148 known feature of human immunity to influenza, characterized by immune memory from

149 intermittent exposure to influenza through vaccination and infection (50-52). To study the
150 response of intranasal vaccination in the context of influenza-specific memory, we established an
151 immunization regimen where C57BL/6 mice were primed intranasally with cold-adapted live-
152 attenuated A/New Caledonia/20/99 (LAIV) which our previous studies have shown to lead to
153 diverse antigen specific responses (**Figure 1A**) (53, 54). After vaccination with LAIV, mice were
154 rested for 30 days to allow the immune response to contract. We focused the subsequent
155 vaccination strategy on influenza NP, a highly expressed internal virion protein that is conserved
156 among diverse influenza strains (55). Recombinant histidine-tagged NP, adjuvant, and NLP were
157 combined as outlined (**Figure 1B**), to generate stable complexes of NP-antigen and cholesterol-
158 tagged CpG bound to the NLP platform, which will be referred to as NLP:NP. Thirty days post-
159 priming, mice were intranasally vaccinated with 5 μ g His-NP and 5 μ g cholesterol-tagged CpG
160 bound to NLP at a molar ratio of 1 NLP : 2.4 NP : 18.4 CpG (**Figure 1A**). NLP stably bound to
161 His-NP at various molar ratios ranging from 2:1 to 16:1 NP to NLP, where formation of non-
162 covalently conjugated NLP:NP complexes was monitored by size exclusion chromatography
163 (SEC) (**Figure 1C**). SEC chromatograms show increasing protein peak intensity as the NP to
164 NLP ratio increases. Additional SEC chromatograms demonstrate that NLP:NP complexes are
165 eluted earlier than either NLP alone or the NLP:CpG complex, indicating that complexes are of a
166 larger size, and stably form conjugates incorporating both antigen and adjuvant. (**Figure 1D**).

167 **Intranasal NLP:NP vaccination selectively boosts NP-specific CD4 T cells**

168 To quantify the CD4 T cell response to NLP:NP immunization regimen, IFN γ and IL-2
169 cytokine ELISpot assays were performed at several time points: the peak of the primary T cell
170 response to LAIV, 30 days post priming, and again following NLP:NP boost. Cells isolated from
171 lung, the lung-draining mediastinal lymph node (mLN), and spleen were used to assess

172 distribution of antigen-specific cells restimulating cells with immunodominant I-A^b restricted
173 peptides from HA, non-structural protein 1 (NS1), NP, and neuraminidase (NA) that were
174 previously defined (56). The primary CD4 T cell response to cold adapted A/New Caledonia/99
175 immunization was assessed seven days post infection (**Figure 2A**). We then evaluated the
176 frequency of influenza-specific CD4 T cells 30 days post-priming and immediately before
177 intranasal NLP:NP boost (**Figure 2B**). Finally, the extent of antigen-specific CD4 T cell
178 boosting was assessed across all three tissues following intranasal NLP:NP immunization
179 (**Figure 2C**). The frequency of IFN γ producing NP-specific CD4 T cells increased 117-fold in
180 lung, 38-fold in mLN, and 100-fold in spleen, relative to LAIV D30 mice. The frequency of IL-2
181 producing cells increased 16-fold, 11-fold, and 60-fold in lung, mLN, and spleen, respectively.
182 Boosting following NLP:NP immunization was only detectable among NP-specific CD4 T cells
183 and not among HA, NS1, or NA-specific CD4 T cells, demonstrating that boosting that is not
184 induced by CpG administration alone. In agreement with this, mice vaccinated with NLP:CpG
185 did not exhibit expansion of NP-specific cells in the lung or periphery (Data not shown).

186 **Intranasal NLP:NP vaccination expands tissue resident CD4 T cell populations in the lung**

187 The localization of CD4 T cells has a profound effect on their protective capacity, and
188 their ability to rapidly respond to infection (12, 22, 27, 31, 57, 58). The microenvironment,
189 including cytokines, chemokines, and antigen-dependent interactions can affect the recruitment,
190 expansion, phenotype, and persistence of the antigen-specific cells. To determine the localization
191 and phenotype of CD4 T cells in the lung elicited by intranasal NLP:NP immunization,
192 intravascular (IV) labeling was employed in conjunction with multiparameter flow cytometry
193 (57). Given the importance of localization within the lung, we were particularly interested in
194 determining if the vaccination protocol elicits tissue-resident CD4 T cell populations in the lung.

195 Using the gating scheme depicted in **Supplementary Figure 1**, live CD4⁺ T cells were
196 partitioned between vasculature (CD45 IV⁺) and tissue (CD45 IV⁻) compartments prior to
197 additional analyses. This enabled assessment of these discreet populations on the basis of
198 antigen-experience, tissue homing potential, and memory precursor potential (59). Intranasal
199 NLP:NP immunization significantly increased both the percentage and abundance of total CD4 T
200 cells localized to the lung tissue relative to that detected in mice only exposed to LAIV (**Figure**
201 **3A-C**). NLP:NP boosted mice had a higher proportion of CD44⁺ CD62L⁻ cells in both tissue
202 and vasculature relative to LAIV alone, as well as a dramatically increased proportion of cells in
203 lung tissue relative to vasculature (**Figure 3D-E**). In terms of total cellular abundance, further
204 analyses revealed that the lung tissue, but not vasculature of NLP:NP immunized mice was
205 enriched for cells of an effector CD44⁺ CD62L⁻ phenotype (**Figure 3F**). Next, we evaluated
206 expression of CD11a, which has been implicated as a critical marker of T cells homing to
207 inflamed tissues and CD69, which plays a role in retention of the recruited lymphocytes at the
208 site of inflammation, especially within non-lymphoid tissues (30, 60-66). NLP:NP immunization
209 significantly increased the abundance of CD11a⁺ CD69⁻ and CD11a⁺ CD69⁺ CD4 T cells in the
210 lung tissue relative to LAIV controls (**Figure 3G-I**). Whereas CD4 T cells uniformly expressed
211 high levels of CD11a, we found substantial heterogeneity in the abundance of cells expressing
212 CD69 (**Figure 3I**). Heterogeneity in CD69 expression may be a consequence of local antigen re-
213 encounter, local cytokine milieu, or reflect positioning within a lung niche that enables CD69-
214 independent retention of CD4 T cells (61, 63, 66). Finally, we examined the relative proportion
215 and overall abundance of short-lived effector Ly6C⁺ cells (59). When expression of Ly6C was
216 examined on tissue resident CD4 T cells, the relative proportion of Ly6C⁺ and Ly6C⁻ negative
217 cells was similar between the two groups, while the abundance of Ly6C⁻ cells was significantly

218 increased in the NLP:NP immunized group (**Figure 3J-L**). These data indicate that intranasal
219 NLP:NP immunization increases the abundance of tissue resident effector CD4 T cells, including
220 those with the potential to persist long term in the lung, and highlights the complexity of CD4 T
221 cell biology in the lung.

222 **NP-specific CD4 T cells are primarily localized to the lung parenchyma as detected by**
223 **pMHC II tetramer staining**

224 While our studies revealed that mice vaccinated by an intranasal route with NLP:NP
225 complexes increased the abundance of lung-localized CD4 T cells, relative to mice that were
226 only primed with LAIV, the antigen specificity of these CD4 T cells was not explicitly defined.
227 To address whether the increased abundance of lung parenchyma localized CD4 T cells are
228 indeed influenza-specific and selectively boosted by administration of NLP:NP, pMHC II
229 tetramers were used in combination with intravascular labeling. In the gating scheme shown in
230 **Supplementary Figure 2**, a double lineage-specific gating scheme was employed to ensure high
231 specificity of pMHC II tetramer staining. When compared to LAIV alone mice, NLP:NP
232 immunized mice display an increased percentage of tetramer positive CD44 high CD4 T cells
233 (**Figure 4A**). The majority of tetramer-positive CD4 T cells are localized to the lung
234 parenchyma, with very few localized to the vasculature (**Figure 4B**). As shown in **Figure 4C**,
235 intranasal NLP:NP immunization significantly increases the abundance of NP-specific CD4 T
236 cells within the lung parenchyma relative to that seen in mice exposed only to LAIV. Mean
237 numbers of tetramer positive CD4 T cells recovered from NLP:NP immunized lung were
238 2.48×10^5 in lung tissue and 5.53×10^3 in lung vasculature. By comparison, mice that were only
239 primed with LAIV had a mean of 2.61×10^3 tetramer positive cells in lung tissue and 8.3×10^1

240 tetramer positive cells in lung vasculature, representing 95-fold and 66-fold increases,
241 respectively (**Figure 4C**).

242 **Co-delivery of antigen and adjuvant on NLP:NPs enhances CD4 T cell responses to** 243 **intranasal vaccination**

244 Given the extent of CD4 T cell boosting observed following intranasal vaccination with
245 NLP:NP, we sought to determine whether the responses are a consequence of co-delivery of
246 antigen and adjuvant on NLP. To address this issue, responses to NLP:NP were analyzed in
247 parallel with delivery of vaccine components separately, but simultaneously, in soluble form at
248 identical doses. Using the vaccination regimen depicted in **Figure 5A**, mice that had been primed
249 with LAIV were subsequently boosted with NLP:NP complexes or soluble NP + CpG. NLP:NP
250 immunization elicited 4.4-fold more IFN γ producing cells and 3-fold more IL-2 producing cells
251 in the lung (**Figure 5B-C**). In the lung draining mLN, there were 3.7-fold more IFN γ producing
252 cells and 1.2-fold more IL-2 producing CD4 T cells were detected (**Figure 5D-E**). NLP:NP
253 immunization elicited 2.3-fold more IFN γ producing cells and 1.5-fold more IL-2 producing
254 cells than soluble NP + CpG immunized mice (**Figure 5F-G**). In both cases, CD4 T cell boosting
255 was only observed among NP-specific cells. Overall, these experiments revealed that NLP:NP
256 immunization elicits a higher frequency of NP-specific CD4 T cells than does soluble antigen
257 and adjuvant at the same dose, and does so in a highly antigen-specific manner.

258 **Intranasal vaccination with NLP:NP elicits a higher frequency of antigen-specific CD4 T** 259 **cells than peripheral vaccination**

260 Given the impact of co-delivery of antigen and adjuvant on the NLP platform, it was
261 important to formally address the impact the route of immunization has on frequency and
262 localization of antigen-specific cells in the lung. In the immunization regimen depicted in **Figure**

263 **6A**, mice were primed with LAIV and boosted with NLP:NP via either an intranasal or
264 subcutaneous (SQ) route. Use of cytokine ELISpot and flow cytometry, showed that intranasal
265 NLP:NP immunization elicits 14.7-fold more NP-specific IFN γ producing cells in the lung than
266 SQ vaccination (**Figure 6B**). Further, intranasal immunization seeded 5.4-fold and 1.9-fold more
267 IFN γ producing cells to mLN and spleen, respectively (**Figure 6C-D**). The frequency of NP-
268 specific CD4 T cells was not significantly different in the popliteal lymph node, which drains the
269 site of subcutaneous immunization (**Figure 6E**). These results indicate the critical role that direct
270 delivery to the respiratory track plays in localizing influenza-specific CD4 T cells in the
271 respiratory tract.

272 The preceding studies also revealed the enhanced overall immunogenicity of intranasal
273 vaccine responses, in both lung and peripheral secondary lymphoid tissues. When the total
274 abundance of antigen-experienced CD44 high CD4 T cells was compared between intranasal and
275 subcutaneous vaccination, intranasal immunization was found to elicit more than 20-fold more
276 CD4 T cells to the lung airway and lung tissue than subcutaneous immunization, as well as
277 leading to a substantial increase in CD4 T cells localized to the lung vasculature (**Figure 6F-H**).
278 Also, representation of cells in the spleen increased almost 2-fold (**Figure 6D**). Lung delivery is
279 critical in seeding tissue resident CD4 T cells that home to the site of subsequent influenza
280 infection, as well as secondary lymphoid tissue.

281 **Intranasal NLP:NP immunization elicits distinct populations of NP-specific CD4 T cells**
282 **localized to the airway, lung parenchyma, and lung vasculature**

283 While we have demonstrated that NLP:NP immunization elicits a population of antigen-
284 specific tissue-resident CD4 T cells, it was important to additionally evaluate whether the
285 vaccine strategy elicits tissue resident T cells that can localize directly to the airways and if so,

286 whether these express markers associated with distinct functional potential from CD4 T cells in
287 other lung compartments. Previous studies of primary and secondary infections have shown that
288 T cells localized to the airways have unique potential to serve as front-line defenders against
289 viral infections, and may have altered functional potential relative to CD4 T cells isolated from
290 other sites within lung or secondary lymphoid organs (67-71). To separately identify lung cells
291 into airway, tissue, and vasculature, we employed an intranasal antibody labeling technique in
292 conjunction with intravascular antibody labeling to definitively identify cells localized to each
293 compartment (70). CD4 T cells isolated from mLN and spleen were examined in parallel.
294 Additionally, CD4 T mice primed only with LAIV were compared for their ability to produce
295 cytokines and degranulate in response to antigen. Shown in the representative gating schemes
296 (**Supplementary Figure 3**), is the strategy used to partition CD4 T cell populations from each of
297 the tissues described above.

298 To address the features of CD4 T cells localized to the three distinct compartments of the
299 lung, cells were restimulated with NP peptide, and stained intracellularly for IFN γ , TNF α , IL-2,
300 and CD107a as a marker for cytotoxic degranulation (72). Employing a Boolean gating strategy
301 to assess polyfunctionality, LAIV-primed mice immunized 30 days prior had very few cytokine
302 producing cells in the lung, with some IFN γ single producers detectable in the BAL and mLN,
303 while IL-2 single producers predominated in the spleen (**Figure 7A**). Applying the same Boolean
304 analyses following NLP:NP boost, NP-specific CD4 T cells from lung and peripheral sites
305 exhibited multifunctionality, producing both IFN γ and TNF α upon restimulation with antigen
306 (**Figure 7B**). A smaller proportion of antigen-specific CD4 T cells simultaneously produce IFN γ ,
307 TNF α , and IL-2, or produce either IFN γ or TNF α alone upon restimulation (**Figure 7B**).
308 Interestingly, a significantly higher frequency of multifunctional CD4 T cells producing IFN γ

309 and TNF α was isolated from the airway relative to lung or peripheral lymphoid tissue (**Figure**
310 **7B**). Due to the increasing evidence that cytotoxic CD4 T cells can contribute to protective
311 immunity to respiratory pathogens, CD4 T cells were examined for markers of cytolytic potential
312 (58, 73-75). We find that vaccination with the NLP:NP conjugate increased the abundance of
313 CD4 T cells in the lung airway and tissue that express the cytotoxic molecule CD107a and the
314 surface marker NKG2, relative to LAIV alone controls (**Figure 7C-D**). Collectively, these
315 results indicate that intranasal delivery of the NLPNP:CPG conjugate poises a population of
316 influenza-specific CD4 T cells that localize to the lung airways and tissue to produce IFN γ ,
317 TNF α , and degranulate upon restimulation with cognate antigen. Airway-localized CD4 T cells
318 display enhanced production of antiviral cytokines and cytotoxic mediators relative to CD4 T
319 cells localized to the lung tissue or vasculature. Further, cells localized to the lung possess
320 enhanced effector functions relative to CD4 T cells isolated from mLN or spleen.

321 **Integrins and chemokine receptor expression among antigen-specific CD4 T cells elicited**
322 **by NLP:NP immunization**

323 Migration of antigen-specific CD4 T cells into the lung is a highly orchestrated process
324 dependent on progressive rolling/tethering mediated by selectins, activation by antigen, and/or
325 chemokines. Firm adhesion and extravasation also depend on integrins (76-81). Once in the
326 tissue, integrins expressed on the newly extravasated T cells can respond to and reinforce signals
327 from other receptors, such as T cell receptor or chemokine receptor. (76-81). Previous studies
328 have implicated integrins $\alpha_1\beta_1$ (CD49a) and $\alpha_2\beta_1$ (CD49b) as critical collagen binding receptors
329 for navigating the collagen-rich microenvironment of the lung, where CD49a interacts with
330 collagen type I and IV, and CD49b interacts with collagen type I. Basement membranes of the

331 airway walls are enriched for collagen type IV and CD49a has been implicated in retention of
332 memory CD8 T cells in proximity to the airways (67, 82).

333 Given the spatially-dependent localization of these types of collagen within the lung, it
334 was of interest to address the expression of integrins of the NLP:NP boosted CD4 T cells, which
335 might modulate their positioning within the lung. Expression of CD49a and CD49b was assessed
336 on antigen-specific CD4 T cells that produced IFN γ and TNF α , the largest subset of cytokine
337 producing cells (**Figure 7B**). These analyses indicated that the majority of antigen-specific CD4
338 T cells across all tissues expressed CD49a, with cells isolated from the airway and lung
339 parenchyma being enriched for CD49a expression (**Figure 8A-B**), with fewer cells expressing
340 CD49b or expressing both integrins (**Figure 7B**). Our results studying the endogenous
341 polyclonal CD4 T cell repertoire responding to this intranasal vaccine are in agreement with
342 previous studies that had implicated CD49a⁺ TCR transgenic CD4 T cells as being important for
343 the early cytokine response following re-exposure to influenza virus (83).

344 In addition to integrin signaling, lung-localized CD4 T cells responding to pathogen
345 infection also received critical positioning cues from chemokine receptors (84-88). No single
346 chemokine receptor has yet been shown to exclusively govern CD4 T cell homing to the lung,
347 suggesting the possibility that multiple chemokine receptors contribute to lung homing or that
348 dominant chemokines vary with the particular immune challenge to the lung (86). We sought to
349 evaluate the chemokine receptor repertoire that may have the potential to contribute to migration
350 from the periphery into the lung parenchyma or into the airway of CD4 T cells induced by the
351 NLP:NP intranasal vaccine. In parallel, chemokine receptor expression was assessed on NP-
352 specific CD4 T cells in the periphery. These experiments revealed that NLP:NP boosted CD4 T
353 cells in lung and peripheral lymphoid organs that produced IFN γ and TNF α were enriched for

354 expression of CXCR3 and CXCR6, whereas expression of putative lung-homing markers
355 CX3CR1, CCR2, and CCR5 was uniformly low (**Figure 8C**). Collectively, these results indicate
356 that integrin CD49a and chemokine receptors CXCR3 and CXCR6 may contribute to positioning
357 of CD4 T cells within the lung.

358 **NLP:NP immunization elicits a population of antigen-specific CD4 T cells that persist in**
359 **the lung long-term**

360 Durability of memory T cells following intranasal vaccination is a high priority for future
361 influenza vaccine platforms (18). Several studies have noted a steady decline of CD4 tissue
362 resident memory within the lung microenvironment after influenza infection (89, 90). This
363 contrasts with other tissues, such as skin or female reproductive tract, where these populations
364 are stably maintained over the long term (30, 60, 61, 63-66, 91). Gradual loss of lung-resident
365 CD4 T cells, potentially through a greater degree of apoptosis within the lung relative to
366 recruitment of cells from the periphery, may underlie decreased protection from reinfection
367 despite the presence of virus-specific CD4 T cells at peripheral sites (69, 92). To assess the
368 durability of the response to intranasal immunization, the abundance of NP-specific CD4 T cells
369 was examined late, at day 270 post-vaccination. Despite attrition of CD4 T cells over time, NP-
370 specific CD4 T cells are still readily detectable in lung, mLN, and spleen at nine months post
371 vaccination (**Figure 9A-C**). The highest frequency of NP-specific CD4 T cells is detected in the
372 lung, suggestive of a stable population that persists long term in the lung or steady recruitment
373 from the periphery to maintain a population of lung localized cells (**Figure 9A**). These findings
374 demonstrate that intranasal NLP:NP immunization elicits a population of influenza-specific CD4
375 T cells that can persist long term in the lung, a critical aspect of any intranasal vaccine candidate.

376 **Intranasal NLP:NP vaccination elicits NP-specific serum and mucosal antibodies**

377 Previous studies have demonstrated that influenza virus infection generates antibodies
378 against the internal proteins of the virus, including NP (93-96). While anti-NP antibodies do not
379 provide sterilizing immunity, studies have demonstrated that prophylactic anti-NP antibody
380 treatment can reduce severity of infection (93). To assess whether intranasal NLP:NP
381 immunization elicits systemic and mucosal antibody responses targeting NP, enzyme-linked
382 immunosorbent assays (ELISA) were performed. Mice were immunized as described previously,
383 and antibody responses to NP were characterized in sera from peripheral blood and the lung
384 mucosa by sampling bronchoalveolar lavage fluid (BALF) (**Figure 10A**). Relative to LAIV
385 alone mice, NLP:NP immunization boosted anti-NP serum antibody titers (**Figure 10B**), as well
386 as IgG and IgA antibody responses in BALF. Delivery of NLP:NP into the respiratory tract
387 elicits significantly more anti-NP IgG and IgA than LAIV alone, suggestive of either antibody
388 transudation from serum or local production of antibody following intranasal immunization
389 (**Figure 10C-D**) (97, 98).

390 **Discussion**

391 In this study, we have evaluated the potential of an intranasal nanolipoprotein vaccine
392 bearing the highly conserved influenza antigen NP to enhance the quantitative representation and
393 potential effector functions of antigen-specific CD4 T cells. We demonstrated that NLP:NP
394 immunization elicited NP-specific CD4 T cells that were localized to three distinct sites within
395 the lung: vasculature, parenchyma, and airway. NP-specific CD4 T cells are also seeded to
396 secondary lymphoid organs. Phenotypic analyses suggested that NLP:NP immunization elicited
397 lung-resident effector CD4 T cell populations, including those with memory precursor potential.
398 Substantiating this memory forming potential, durable lung-localized CD4 T cell responses were

399 found to persist at least nine months post-vaccination. Importantly, NLP vaccination potentiated
400 a population of airway-localized NP-specific CD4 T cells that responded robustly to
401 restimulation with cognate antigen via production of antiviral cytokines and degranulation, and
402 did so at a frequency higher than lung tissue, lung vasculature, or peripheral CD4 T cells. Lung
403 tissue and vasculature localized cells also had enhanced effector functions relative to NP-specific
404 CD4 T cells isolated from mLN or spleen. These lung localized CD4 T cell responses were
405 found to be dependent on co-delivery of NP and CpG on the NLP platform, and immunization by
406 the intranasal route. Additionally, intranasal NLP:NP vaccination was also found to elicit
407 mucosal antibody responses.

408 There were a number of notable features of the immunity generated by intranasal
409 NLP:NP vaccination. A critical subset of CD4 T cells elicited by NLP:NP vaccination were
410 airway-homing CD4 T cells that produced IFN γ , as airway-localized CD4 and CD8 T cells have
411 been implicated in protection from diverse viral infections such as SARS, RSV, Sendai virus,
412 and influenza virus (67-71). Further, airway-localized CD4 T cells have been shown to mediate
413 protection from virus infection after direct intratracheal transfer into naïve mice, and have the
414 potential to do so in a CD8-independent manner (70). Another defining feature of the
415 multifunctional CD4 T cells elicited by NLP:NP immunization is production of TNF α , which
416 has been implicated as a critical mediator for limiting influenza virus replication in lung
417 epithelial cells (99). Finally, airway-localized CD4 T cells elicited by NLP:NP vaccination
418 display increased cytotoxic potential relative to lung tissue, parenchyma, or periphery, as
419 measured by degranulation in response to antigen and expression of surface marker NKG2.
420 Cytotoxic CD4 T cells expressing NKG2 have previously been implicated in protection from
421 influenza infection through MHC-II restricted killing via Granzyme B and CD107a (58). This

422 result is in contrast to airway-homing CD8 T cells, which have inferior cytolytic capacity relative
423 to lung tissue or vasculature localized CD8 T cells (100).

424 Positioning of antigen-specific CD4 T cells depends on complex integrated positioning
425 cues mediated by the interactions of selectins, integrins, chemokine receptors, and T cell
426 receptors within the microenvironment of the lung (76-81). We found that the majority of
427 cytokine producing cells elicited by NLP:NP vaccination expressed integrin CD49a, which is
428 known to interact with collagen type I and IV, a prominent component of basement membranes
429 in the lung, and contributes to positioning of T cells near the airway (67, 82). Our experiments
430 indicated that NP-specific CD4 T cells elicited by intranasal NLP:NP vaccination expressed
431 chemokine receptors CXCR3 and CXCR6. CXCR3 interacts with CXCL9, CXCL10, and
432 CXCL11, and serves an important role in migration of activated Th1 cells and amplification of
433 IFN γ -mediated downstream signaling (101). Further, expression of CXCR3 in the LN and spleen
434 is thought to serve as a potential marker of memory T cells that can participate in recall
435 responses to viral reinfection (101). CXCR6 interacts exclusively with CXCL16, which previous
436 studies have shown to be constitutively expressed in the lung by airway epithelial cells. CXCL16
437 was found to be co-localized with EpCam, which has known binding activity to integrin β_1 (102).
438 While our studies have not yet implicated a single chemokine receptor that drives CD4 T cell
439 migration to the lung after intranasal vaccination, signals through cell surface integrins and
440 chemokine receptors may synergize to recruit protective CD4 T cells into the lung and airway
441 (86).

442 The vaccine platform characterized in our studies presents a wide array of advantages
443 relative to other platforms. NLPs are non-toxic, intrinsically non-immunogenic, stable under
444 biological conditions, self assembling, and allow for flexible conjugation (41, 47-49). Owing to

445 their flexibility and robust immunogenicity, other lipid nanoparticle vaccine candidates are in
446 clinical trials as a COVID-19 vaccines (103). To increase the translational potential of the NLP
447 platform, minimal modifications could be made to the transition metal-chelating lipids and co-
448 delivered adjuvant on the nanoparticles. Like Ni^{2+} , transition metals such as Cu^{2+} and Zn^{2+} allow
449 for formation of coordination bonds via interactions on the imidazole ring of histidine residues,
450 but without the potential for induction of innate immune responses through cross-linking of
451 human TLR4 (104, 105). Additionally, by employing adjuvants with demonstrated safety
452 profiles in humans, such as the delta inulin-based adjuvant Advax®, robust vaccine responses
453 could be achieved without dangerous adverse reactions to vaccination (106). Given these
454 modifications, the NLP platform could play an important role in pandemic preparedness.

455 In contrast to alternative vaccine platforms which can elicit potentially pathogenic Th17
456 polarized cell (45, 107, 108), intranasal NLP vaccination elicits CD4 T cells that produce
457 prototypical type-1 biased cytokines, which have been shown to be most protective against
458 intracellular pathogens like influenza virus. CD4 T cells elicited by intranasal immunization
459 express markers associated with tissue residency in the lung, a subsets of cells shown to be
460 critical for early control of viral infection. The progressive loss of lung tissue-resident memory
461 populations (69, 92) has emphasized the importance of durable responses in vaccine design
462 efforts (18). NP-specific CD4 T cells in the lung at the peak of T cell response were found to
463 lack expression of Ly6C, a putative marker of memory precursor cells. Previous studies have
464 shown that Ly6C-low CD4 T cells are longer lived and have greater proliferative potential in
465 response to re-infection than Ly6C high CD4 T cells, a critical parameter for cells elicited by
466 vaccination that can respond to subsequent infections (59). Our data from nine months post-
467 vaccination demonstrated the durability of CD4 T cell responses to NLP vaccination, whereas

468 other reports have found that antigen specific CD4 T cell responses were found to be
469 undetectable two months after the peak of the response (69). Collectively, these studies
470 emphasize the key feature of CD4 T cells specific for the conserved vaccine antigen NP that can
471 be induced by intranasal protein vaccination with a novel nanoparticle platform. The localization,
472 polyfunctionality, and persistence *in vivo*, coupled with the multiplicity of function that memory
473 CD4 T cells are known to convey (23-26), suggest the validity of pursuing intranasal vaccine
474 approaches for induction of broadly protective immunity that can rapidly respond to influenza
475 virus challenge.

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491 **Materials and methods**

492 **Animals and infections.** Female C57BL/6 mice were obtained from the National Cancer
493 Institute and maintained at a specific-pathogen free facility at the University of Rochester
494 Medical Center according to institutional guidelines. Mice were used at 8-10 weeks of age.

495 **Ethics statement.** All mice were maintained under specific-pathogen-free conditions at the
496 University of Rochester Medical Center according to institutional guidelines. All animal
497 protocols adhere to AAALAC International, the Animal Welfare Act, the PHS Guide, and were
498 approved by the University of Rochester Committee on Animal Resources, Animal Welfare
499 Assurance Number A3291-01. The protocol under which the studies were conducted was first
500 approved March 4, 2006 (protocol 2006-030) has been reviewed and re-approved every 36
501 months with the most recent re-approval January 23, 2018.

502 **NLP formulation and immunization**

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504 **Intravascular labeling.** Mice were anaesthetized via inhalation of isoflurane and retro-orbitally
505 injected with 3 μ g anti-mouse CD45 (30-F11, Tonbo) in a total volume of 100 μ L. Three
506 minutes following IV injection, peripheral blood was collected by cardiac puncture into tubes
507 containing sodium heparin (30 USP units per mL).

508 **Tissue processing and cell isolation.** Lung and lymphoid tissues were excised from euthanized
509 mice. Lung tissues were minced using surgical scissors in media containing collagenase type II
510 or IV (10^3 units/mL) and DNase I (30 μ g/mL) in RPMI supplemented with 2.5% fetal bovine
511 serum (FBS) and 10 mM HEPES. Individual lungs were added to GentleMACS tubes (Miltenyi
512 130-093-237) and processed on GENTLEMACS setting Lung 01, incubated for 1 hour at 37°C
513 with constant shaking, then processed on setting Heart 01. Lung tissues were subsequently

514 passed through 40 μ M sterile nylon mesh and rinsed with Dulbecco's modified Eagle medium
515 (DMEM, Gibco) supplemented with 1% gentamycin and 10% heat-inactivated FBS. The lymph
516 node was disrupted using 40 μ M sterile nylon mesh and a 5 mL syringe plunger. Resulting single
517 cell suspensions were treated with ACK lysis buffer (0.15 M NH_4Cl , 1.0 mM KHCO_3 , 0.1 mM
518 NaEDTA, pH 7.2) to deplete red blood cells.

519 **Flow cytometry.** For surface staining experiments, 2×10^6 cells were added to a U-bottom
520 plate. Cells were washed twice with PBS, then incubated with fixable live/dead aqua (Life
521 Technologies) for 30 minutes at 4°C according to manufacturer's instructions. Cells were
522 subsequently washed twice with FC stain buffer (Corning PBS plus 2% heat-inactivated FBS and
523 0.01% sodium azide) and resuspended in anti-mouse CD16/CD32 (BD FC block, 2.4G2) for 20
524 minutes at 4°C. Without washing, cells were stained for 30 minutes at 4°C with the following
525 antibodies: CD4 (RM4-5, BD Biosciences), CD8 (5H10, Biolegend), CD11a (2D7, BD
526 Biosciences), CD44 (IM7, Tonbo), CD49a (Ha31/8, BD Biosciences), CD49b (Ha1/29, BD
527 Biosciences), CD49d (R1-2, BD Biosciences), CD62L (MEL-14, Biolegend), and CD69
528 (H1.2F3, Biolegend). Data were acquired using a BD LSR-II, configured with 488 nm, 633 nm,
529 407 nm, and 532 nm lasers.

530 **Intracellular cytokine staining.** Cells from LAIV primed and NLP:NP vaccinated mice were
531 isolated, and co-cultured in U-bottom 96 well plates (3×10^5 cells/well) with splenocytes from
532 naïve syngenic donors (5×10^5 cells/well) with or without NP-derived peptide. Brefeldin A,
533 monensin, and anti-CD107a antibody (1D4B) were added to cultures 2 hours after stimulation,
534 then incubated for an additional 6 hours for a total of 8 hours. Plates were stored overnight at
535 4°C. Cells were washed and surface stained as described above. Cells were fixed and
536 permeabilized using the eBioscience FoxP3 transcription factor staining kit according to

537 manufacturer's instructions. Cells were stained with the following antibodies IFN- γ (XMG1.2,
538 BD Biosciences), IL-2 (JES6-5H4, BD Biosciences), TNF α (MP6-XT22, Biolegend) for 1 hour
539 at 4°C.

540 **MHC-II tetramer staining and enrichment.** Lung single cell suspensions were generated as
541 described above. Cells were then stained with 10 nM NP261:I-A^b-streptavidin-phycoerythrin
542 tetramer for 1hour at room temperature in diluted FC block (2.4G2 from BD) as described (109).
543 Cells were then washed with FC stain buffer. Tetramer stained cells were resuspended in a
544 volume of 200 μ L and 50 μ L of anti-PE microbeads (MACS 130-048-801) for 30 minutes at
545 4°C. Cells were washed with FC stain buffer and resuspended in 3 mL FC stain buffer before
546 being passed over LS columns (MACS 130-042-401). The column was washed three times with
547 3 mL of FC stain buffer. The column was removed from the magnetic field and the bound
548 fraction was eluted in 5 mL by plunging the column. The resulting column-bound and unbound
549 fractions underwent viability staining and surface staining as described above. Cells were stained
550 with a cocktail containing antibodies specific for B220 (RA3-6B2), CD11b (M1/70.15), CD11c
551 (N418), F4/80 (BM8), CD3 (145-2C11), CD4 (RM4-5), CD8 (5H10), and CD44 (IM-7). The
552 entire column-bound fraction was collected and surface stained as described above.

553 **ELISpot Assay.** The 96-well filter plates (Millipore, Billerica, MA, USA) were coated with 2
554 μ g/mL purified rat anti-mouse IL-2 (JES6-1A12, BD) or IFN γ (AN-18, BD) in PBS overnight at
555 4°C. Prior to plating, wells were washed with media to remove unbound antibody, and incubated
556 with media for 1 hour at room temperature to block non-specific binding. CD4 T cells (200,000
557 mLN/spleen cells or 50,000 lung cells) were purified by negative selection (MACS 130-104-
558 454) and co-cultured with 500,000 syngeneic splenocytes and 5 μ M peptide in a total volume of
559 200 μ L for 16-18 hours at 37°C with 5% CO₂. Cells were subsequently removed from the filter

560 plates, and washed with ELISpot wash buffer (1X PBS with 0.1% Tween-20). Biotinylated rat
561 anti-mouse IL-2 (JES6-1A12) or IFN γ (XMG1.2) was diluted to 2 μ g/mL in ELISpot wash
562 buffer supplemented with 10% FBS in a volume of 50 μ L for 30 minutes at room temperature.
563 Plates were washed with ELISpot wash buffer, and streptavidin-conjugated alkaline phosphatase
564 (Jackson Immuno Research, West Grove, PA, USA) was added at a 1:1000 dilution in ELISpot
565 wash buffer supplemented with 10% FBS and incubated for 30 minutes at room temperature.
566 Plates were washed with ELISpot wash buffer and incubated with Vector Blue substrate kit III
567 (Vector Laboratories, CA, USA) in 100 mM Tris (pH 8.2) for five minutes at room temperature.
568 Following development, plates were washed with water and dried. Quantification of spots was
569 performed using an Immunospot reader series 5.2 with Immunospot software version 5.1.

570 **ELISA Assay.** ELISA assays were performed as described previously (110). Sera were collected
571 from individual mice and used to measure NP specific antibody. Recombinant NP was A/New
572 Caledonia/20/99 was produced as described previously. The 96-well polystyrene flat bottom
573 plates were coated overnight with 200 ng/100 μ L NP at 4°C. Wells were washed with ELISA
574 wash buffer (1X PBS with 0.05% Tween 20) and incubated with 3% BSA in PBS for 1 hour at
575 room temperature to block non-specific binding. Blocking buffer was removed and serial 4-fold
576 dilutions (in 0.5% BSA-PBS) of serum samples were incubated for 2 hours at room temperature.
577 Wells were washed with ELISA wash buffer, then incubated with 1:1000 alkaline phosphatase-
578 conjugated goat anti-mouse Ig (Southern Biotech, Birmingham, AL, USA) in 1% BSA-PBS. *P*-
579 nitrophenyl phosphate substrate (Sigma) was dissolved in diethanolamine substrate buffer (0.33
580 mg/mL) and 100 μ L was added per well. Plates were developed at room temperature for 40
581 minutes, and absorbance was reach at 405 nm using SoftMax Pro software and a VMax plate
582 reader.

583 **Statistical analyses.** Statistical analyses were performed using GraphPad Prism software version
 584 8.4.3 (GraphPad Software, San Diego, CA). Significance (*, P<0.05; **, P<0.01; ***, P<0.001;
 585 ****, P<0.0001) for comparisons of cellular frequency and abundance between LAIV and
 586 LAIV+NLP boost cohorts was determined by two-way ANOVA with Tukey's correction for
 587 multiple comparisons or unpaired, two-tailed t test with Welch's correction. The test performed
 588 is indicated in the figure legend.

589 **Synthetic peptides.** Seventeen-mer peptides overlapping by 11 amino acids encompassing the
 590 entire sequence of viral proteins were obtained from the NIH Biodefense and Emerging
 591 Infectious Disease Research Repository, NIAID, NIH. Individual peptides were reconstituted
 592 and used at a final concentration of 5 μ M. Sequences of peptides used in this study are listed
 593 below.

I-A^b-restricted epitopes	
Epitope	Sequence
HA 16	90 KESWSYIVETPNPENG 106
HA 35	203 NQRALYHTENAYVSVVS 219
NS1 19	108 KQKVAGPLCVRMDQAIM 124
NP45	261 RSALILRGSVAHKSCLP 277
NA 30	171 NSKFESVAWSASACHDG 187

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602 **Disclosure**

603 The authors declare no conflicts of interest.

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931 **Supporting information**

932 **Figure 1: Potentiating lung localized CD4 T cell immunity via intranasal immunization**

933 (A) Schematic representation of immunization regimen. Naïve C57BL/6 mice were immunized
934 with monovalent live attenuated influenza virus (LAIV), a 6:2 reassortant of caA/Ann
935 Arbor/6/60 and A/New Caledonia/99. Responses were allowed to contract for 30 days prior to
936 intranasal boost with NLP. NLP immunizations were comprised of 5 µg recombinant influenza
937 NP and 5 µg CpG conjugated to the NLP at a molar ratio of 1:2.4:18.4, NLP to NP to CpG,
938 respectively. Responses were analyzed 10 days post boost.

939 (B) Schematic demonstrating the NLP self-assembly process. Purified components are
940 solubilized with surfactant and mixed in aqueous solution. Removal of the surfactant initiates
941 self-assembly of the functionalized NLPs. Conjugation of adjuvants and antigens is achieved by
942 reacting with cognate NLP surface functionalities (e.g. His-tag with chelated nickel) or through
943 anchoring of lipidic moieties featured on amphiphilic cargo molecules (e.g cholesterol-tagged
944 CpG).

945 (C) NLPs biomimics of endogenous HDL, where a lipid raft of dipalmitoylphosphatidylcholine
946 (DOPC) and Ni-chelating lipid is stabilized by apolipoprotein. Binding of a diverse array of
947 antigens and adjuvants is facilitated by this vaccine design. Cholesterol-tagged CpG and
948 histidine-tagged influenza nucleocapsid protein were used throughout these experiments. SEC
949 demonstrates the non-covalent conjugation of His-tagged proteins (blue traces) with nickel-
950 chelating NLPs (black traces) to form protein:NLP complexes (red traces). SEC can be used not
951 only for analysis, but also for purification of the NLP complexes.

952 (D) SEC demonstrates the incorporation of both NP protein and cholesterol modified CpG in the
953 final vaccine assembly. Incorporation of adjuvant (e.g. cholesterol-tagged CpG) into the NLP is

954 readily monitored by SEC. For example, increasing ratios of CpG results in increased absorbance
955 and decreased retention time of the NLP construct.

956 **Figure 2: Intranasal NLP:NP:CpG immunization boosts NP-specific CD4 T cells in the**
957 **lung**

958 (A) Naïve C57BL/6 mice were primed with live attenuated influenza virus to establish influenza-
959 specific memory. The CD4 T cell response was assayed in lung, mLN, and spleen 7 days post
960 infection via IL-2 and IFN γ cytokine ELISpot to identify immunodominant CD4 T cell epitopes
961 across HA, NS1, NP, and NA viral proteins.

962 (B) Thirty days post immunization with LAIV, CD4 T cell responses were assayed in lung,
963 mLN, and spleen via IL-2 and IFN γ cytokine ELISpot to characterize the immune response prior
964 to intranasal boost with NLP:NP:CpG.

965 (C) Ten days post intranasal immunization with NLP:NP:CpG, the extent of CD4 T cell boosting
966 in lung, mLN, and spleen was quantified by IL-2 and IFN γ cytokine ELISpot.

967 Results are represented as the mean number of cytokine-producing spots per million CD4 T cells
968 with standard deviation shown. Mean is representative of three to five independent experiments
969 of five pooled mice each.

970 **Supplementary Figure 1: Representative gating scheme for tissue resident CD4 T cells in**
971 **murine lung**

972 (A) Flow cytometry gating scheme used to identify CD4 T cell subsets following LAIV priming
973 and NLP:NP boost. Cellular aggregates and dead cells were excluded, prior to gating on CD4 T
974 cells. CD4 T cells were subsequently partitioned between lung tissue and vasculature, prior to
975 gating on CD44 and CD62L to assign cells to naïve (CD44⁻ CD62L⁺) or effector (CD44⁺

976 CD62L-) phenotypes. Effector CD4 T cells were gated for their expression of CD11, CD69, and
977 Ly6C.

978 **Figure 3: Intranasal NLP:NP:CpG increases the frequency and abundance of tissue**
979 **resident CD4 T cells relative to LAIV alone**

980 (A) Analysis of lung localization among CD4 T cells, depicting representative flow plots, (B) the
981 frequency of lung tissue-localized CD4 T cells, and (C) absolute abundance of CD4 T cells in the
982 intravascular labeled CD45+ lung vasculature and unlabeled CD45- lung tissue.

983 (D) CD4 T cells were assessed for their expression of CD44 and CD62L. (E) The frequency and
984 (F) absolute abundance of effector CD44+ CD62L- CD4 T cells was assessed in the lung tissue
985 and vasculature.

986 (G) CD44+ CD62L- effector CD4 T cells were subsequently gated on expression of CD11a and
987 CD69. (H) The frequency and (I) abundance of CD11a+ CD69+/- was calculated among effector
988 CD4 T cells in the lung tissue.

989 (J) Expression of memory-precursor marker Ly6C was assessed among CD44+ CD62L- effector
990 CD4 T cells. (K) The frequency and (L) abundance of Ly6C+ and Ly6C- CD4 T cells was
991 assessed among lung tissue localized effector CD4 T cells.

992 Results are represented as the mean number of the respective population with standard deviation
993 and individual mice shown. Mean is representative of three independent experiments of five
994 individual mice each. With the exception of (B), significance (***, $P < 0.001$; ****, $P < 0.0001$)
995 for comparisons of cellular frequency and abundance between LAIV and LAIV+NLP boost
996 cohorts was determined by two-way ANOVA with Tukey's correction for multiple comparisons.
997 Unpaired, two-tailed t test with Welch's correction was performed in (B).

998 **Supplementary Figure 2: Representative gating scheme for pMHCII tetramer staining**

999 (A) Gating scheme used to address whether the tissue localized CD4 T cells boosted by
1000 vaccination were antigen specific. Live, single cells were then gated using a lineage exclusion
1001 gate (CD11b, CD11c, F4/80, B220) to ensure specific detection of tetramer specific events
1002 within antigen experienced CD44⁺ Tcrβ⁺ CD4⁺ T cells. Tetramer⁺ cells were then partitioned
1003 by tissue and vasculature localization.

1004 **Figure 4: Intranasal NLP:NP immunization boosts antigen specific CD4 T cells which**
1005 **localize to the lung parenchyma**

1006 (A) Representative plot of NP-specific CD4 T cells detected by I-A^b pMHC-II tetramer staining
1007 in LAIV and LAIV+NLP immunized murine lung.

1008 (B) Frequency of vasculature localized CD45⁺ and tissue localized CD45⁻ pMHC-II tetramer⁺
1009 cells.

1010 (C) Quantification of the total number of NP-specific CD4 T cells within the lung tissue and
1011 vasculature of LAIV and LAIV+NLP immunized mice.

1012 Results are represented as the mean number of pMHC-II tetramer⁺ cells per lung with standard
1013 deviation and individual replicates shown. Mean is representative of 10 individual mice analyzed
1014 as pools of two lungs each. Cell numbers from pooled lungs are divided by two to reflect the
1015 cellularity of a single lung. Significance (***, P<0.001) for comparisons of cellular abundance
1016 between LAIV and LAIV+NLP boost cohorts was determined by two-way ANOVA with
1017 Tukey's correction for multiple comparisons.

1018 **Figure 5: Immunization with NLP:NP:CpG complexes elicits a higher frequency of**
1019 **antigen-specific CD4 T cells in the lung than soluble NP and CpG alone**

1020 (A) Schematic representation of the immunization regimen, where mice were primed with LAIV
1021 and subsequently boosted with NLP:NP:CpG or soluble NP+CpG, and responses were assayed
1022 10 days post intranasal boost.

1023 The CD4 T cell response to NLP:NP and soluble NP+CpG immunization was compared via
1024 IFN γ and IL-2 cytokine ELISpot assay in lung (B-C), mLN (D-E), and spleen (F-G).

1025 Results are represented as the mean with standard deviation of two independent experiments of 5
1026 pooled mice each. Statistical significance was determined by unpaired, two-tailed t test with
1027 Welch's correction.

1028 **Figure 6: Intranasal immunization with NLP:NP:CpG elicits a higher frequency of lung**
1029 **and mLN localized antigen specific CD4 T cells relative to peripheral immunization with**
1030 **NLP:NP:CpG**

1031 (A) Schematic representation of immunization regimen used to compare the effect of intranasal
1032 (IN) vs. subcutaneous (SQ) immunization with NLP:NP.

1033 Comparison of the frequency of (B) lung, (C) mLN, and (D) spleen localized CD4 T cell
1034 responses following IN or SQ immunization as determined by cytokine ELISpot.

1035 Assessment of the abundance of antigen-experienced CD4 T cells within the (F) airway, (G) lung
1036 tissue, and (H) lung vasculature following IN or SQ NLP:NP immunization.

1037 Results are represented as the mean with standard deviation of four individual mice per cohort.

1038 One cohort was assessed by cytokine ELISpot and a second cohort was assessed by flow
1039 cytometry. Statistical significance was determined by unpaired, two-tailed t test with Welch's
1040 correction.

1041 **Supplementary Figure 3: Representative gating scheme for BAL, Lung, mLN, and spleen**
1042 **to assess the functional potential of antigen specific CD4 T cells at each site**

1043 (A) Representative gating scheme of cytokine producing cells isolated from airway. Live, single
1044 cells were gated first on CD4, then on intranasal antibody labeled CD44⁺ cells, followed by
1045 Boolean combination gating on IFN γ , TNF α , and IL-2.

1046 (B) Following the scheme shown above, live single cells were gated on CD4 and CD44. Cells
1047 were then gated on intravenous antibody labeling to partition lung into vasculature and tissue
1048 prior to Boolean combination gating on IFN γ , TNF α , and IL-2.

1049 (C) The same gating strategy was employed for mLN and spleen, but without use of a gate to
1050 assign intravascular labeling status.

1051 **Figure 7: Intranasal NLP:NP immunization elicits a polyfunctional subset of antigen**
1052 **specific CD4 T cells which localize to distinct compartments of the lung**

1053 CD4 T cells were restimulated with cognate antigen and stained intracellularly with IFN γ , TNF α ,
1054 and IL-2 antibodies. Cells producing one or more cytokines were determined using Boolean
1055 gating. The frequency of cells producing one or more cytokines was quantified among CD4 T
1056 cells from LAIV alone (A) or from LAIV+NLP boosted (B) mice were assessed.

1057 Quantification of antigen experienced CD4 T cells that underwent degranulation (C) in response
1058 to antigen stimulation or those expressing putative cytolytic marker NKG2 (D).

1059 **Figure 8: Antigen specific CD4 T cells elicited by NLP:NP:CpG immunization are enriched**
1060 **for expression of integrin CD49a and chemokine receptors CXCR3 and CXCR6**

1061 (A) Representative flow plots of integrin CD49a and CD49b among IFN γ and TNF α producing
1062 CD4 T cells across the indicated tissues.

1063 (B) Quantification of CD49a and CD49b by IFN γ and TNF α producing CD4 T cells
1064 Expression of chemokine receptors CX3CR1, CXCR3, CXCR6, CCR2, and CCR5 by IFN γ and
1065 TNF α producing CD4 T cells.

1066 **Figure 9: NLP:NP:CpG immunization elicits a population of antigen-specific CD4 T cells**
1067 **that persist in the lung and secondary lymphoid organs long-term following intranasal**
1068 **administration**

1069 Persistence of antigen specific CD4 T cells was assessed 9 months post immunization to assess
1070 durability of responses without confounding effects of immunosenescence. The frequency of IL-
1071 2 and IFN γ producing cells was assessed in lung (A), mLN (B), and spleen (C) by ELISpot
1072 assay. Results, represented as cytokine producing spots per million CD4 T cells, are
1073 representative of three independent experiments of five pooled mice each.

1074 **Figure 10: Intranasal NLP:NP:CpG immunization boosts NP-specific serum and mucosal**
1075 **antibody responses**

1076 (A) LAIV primed and LAIV+NLP boosted mice were assayed for NP-specific serum and
1077 mucosal antibody responses by harvesting serum and bronchoalveolar lavage fluid (BALF) at the
1078 indicated time point.

1079 (B) Serum IgG responses against NP were assessed in immunized and naïve mice via ELISA.

1080 (C) Mucosal IgG and (D) Mucosal IgA targeting NP were assessed in BALF via ELISA.

1081 Data were plotted as arbitrary units (AU) by converting the serum dilution within the treatment
1082 groups that achieved the same detectable reactivity on the linear portion of the antibody titration
1083 curve. Data are represented as mean of 12 individual mice per treatment group with standard
1084 deviation shown. Statistical significance was determined by unpaired, two-tailed t test with
1085 Welch's correction.

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