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Using gold nanoparticles for enhanced intradermal delivery of poorly soluble auto-antigenic peptides ☆☆☆☆☆

R.K Singh^{a,1}, C. Malosse^b, J. Davies^a, B. Malissen^{b,c}, E. Kochba^d, Y. Levin^d, J.C Birchall^e, S.A Coulman^e, J. Mous^{f,2}, M.A McAteer^f, C.M Dayan^{a,*}, S. Henri^b, F.S Wong^a

^aDivision of Infection & Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, UK

^bCentre d'Immunologie de Marseille-Luminy, Aix Marseille Université, Inserm, CNRS, Marseille, France

^cCentre d'Immunophénomique, Aix Marseille Université, INSERM, CNRS, Marseille, France

^dNanoPass Technologies Ltd., Nes Ziona, Israel

^eSchool of Pharmacy and Pharmaceutical Sciences, Cardiff University, UK

^fMidatech Pharma PLC, Cardiff, UK

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Abstract

Ultra-small 1-2 nm gold nanoparticles (NP) were conjugated with a poorly-soluble peptide auto-antigen, associated with type 1 diabetes, to modify the peptide pharmacokinetics, following its intradermal delivery. Peptide distribution was characterized, in vivo, after delivery using either conventional intradermal injection or a hollow microneedle device. The poorly-soluble peptide was effectively presented in distant lymph nodes (LN), spleen and draining LN when conjugated to the nanoparticles, whereas peptide alone was only presented in the draining LN. By contrast, nanoparticle conjugation to a highly-soluble peptide did not enhance in vivo distribution. Transfer of both free peptide and peptide-NPs from the skin to LN was reduced in mice lacking lymphoid homing receptor CCR7, suggesting that both are actively transported by migrating dendritic cells to LN. Collectively, these data demonstrate that intradermally administered ultra-small gold nanoparticles can widen the distribution of poorly-soluble auto-antigenic peptides to multiple lymphoid organs, thus enhancing their use as potential therapeutics.

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Key words: Gold nanoparticles; Hydrophobic; Peptide; Intradermal; Microneedle; Autoantigen

Type 1 diabetes is an autoimmune disease characterized by the destruction of insulin-producing beta cells within the pancreas.¹ Insulin maintains blood glucose levels but is not a cure. Hence, we seek strategies to deliver antigen-specific immunotherapy to reduce immune-mediated beta cell damage.

The skin is an accessible route for therapeutic delivery. The rich network of antigen-presenting cells, including epidermal Langerhans and dermal dendritic cells (DCs) in skin, facilitates both immune activation and regulation, upon delivery of a therapeutic.²⁻⁵ Microneedles (MNs) are a viable alternative to

conventional hypodermic needles for intradermal (ID) delivery, being both minimally invasive and relatively pain-free.^{6,7} A variety of MNs exists, including the 600 µm hollow MNs (MicronJet600™), which have been used in this study and in the clinical setting to deliver insulin,⁸ influenza vaccine,^{9,10} zoster vaccine¹¹ and polio.^{12,13} MicronJet600™ hollow MNs reproducibly and effectively deliver material into the dermal compartment, with a significant vaccine dose-sparing effect and improved immunogenicity, compared with intramuscular and subcutaneous delivery.¹⁴

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☆☆ **Conflicts of interest:** none

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*Corresponding author at: Division of Infection & Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, UK.

E-mail address: dayancm@cardiff.ac.uk. (C.M. Dayan).

¹ **Present address Department of Oncology, University of Oxford, Oxford, UK

² Present address MRM Consulting GmbH, Switzerland.

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Nanoparticles (NPs) can be conjugated to multiple therapeutics, including peptides, as drug delivery agents. They can protect peptides from degradation, and can form a depot at the site of injection so enhancing antigen retention and uptake by DCs. Moreover, small carbohydrate-coated gold NPs also offer reduced toxicity¹⁵ and bio-compatibility as well as exhibiting anti-inflammatory properties.¹⁶ Furthermore, gold (Au) can be easily and covalently decorated on its surface by exploiting the strong soft-soft interaction between Au atoms and sulfur. Therefore, gold NPs represent a versatile nanoplatform for development of epitope-based vaccines.¹⁷

Recently, NPs have been used to prevent autoimmune diabetes in the non-obese diabetic (NOD) mouse model of diabetes by generating tolerogenic DCs and promoting T regulatory cell expansion to re-establish immune tolerance.^{18,19} Although promising, both studies utilized systemic (intravenous or intraperitoneal) delivery systems, as well as large (>50 nm) NPs. In humans, skin-mediated delivery is more practical and convenient, has fewer safety concerns and can exploit the local immune cells. We have recently demonstrated that gold NPs (Midacore™) (<5 nm hydrodynamic radius), conjugated to a peptide autoantigen, can be effectively delivered into the dermal and epidermal layers of human skin explants by MicronJet600™ hollow MNs and target local antigen-presenting cells.²⁰ This present study is the first published report exploring the in vivo pharmacokinetics and resulting systemic immune response following MN-mediated delivery of a poorly-soluble auto-antigenic peptide conjugated to ultra-small gold NPs.

Methods

Animal models

BDC2.5 TCR transgenic NOD mice²¹ have diabetogenic CD4+ T-cells that recognize a hybrid insulin-chromogranin A peptide (HIP)²² and mimotope peptides (the specific one used in this study is designated BDC2.5mimotope peptide).²³ The mice were purchased from the Jackson laboratory and have been bred in a specific pathogen-free facility of Cardiff University. OTII TCR transgenic C57BL/6J mice²⁴ and CCR7^{-/-} mice²⁵ on the C57BL/6J genetic background have previously been described. C57BL/6J mice were purchased from Janvier (France). Mice were maintained in individually-ventilated filter cages in scintainers on a 12 h light/dark cycle. Animal procedures were approved by University ethical review committee. All procedures relating to NOD mice were performed in accordance with protocols approved by the UK Home Office. All procedures relating to OTII and CCR7^{-/-} mice were carried out in accordance with French and European directives.

Peptides and solubility

The peptides (physicochemical properties shown in Table 1) were purchased from Peptide Synthetics, manufactured to >95% purity: BDC2.5mimotope – YVRPLWVRME; Hybrid Insulin Peptide (HIP) – DLQTLALWSRMD; Ovalbumin323-339 peptide (OTII)-ISQAVHAAHAEINEAGR.

NP synthesis/characterization

Gold NPs (Midacore™) with a gold core size of <5 nm were synthesized and supplied by Midatech Pharma.²⁰ NPs were conjugated with each of the poorly-soluble HIP and BDC2.5mimotope peptides as well as the highly-soluble ovalbumin peptide, OTII, using custom-synthesized peptides with a thiol propionic acid linker, in an amide linkage at the N terminal (–S(CH₂)₂–CONH) (AmbioPharm Inc., North Augusta, SC, USA). Figure 1 illustrates the schematic structure of NPs covalently linked to BDC2.5mimotope peptide and Table 2 summarizes the NP properties. For all reactions, gold (III) chloride was mixed with a 3-fold excess of organic ligands and peptide in different ratios (5% β glucose C2 (synthesized in-house) and either 94% or 92% L-glutathione oxidized (Sigma Aldrich) and 1% or 3% peptide). Glutathione enabled non-enzymatic intracellular activation and release. The NPs were produced by reduction, following rapid addition of a 20-fold molar excess, relative to gold, of freshly-made 1 M sodium borohydride (Sigma Aldrich, Poole, UK) under vigorous vortex mixing. The samples were continuously vortexed for 1 min followed by a further 1 h constant mixing on a flatbed shaker at room temperature. After 1 h, the NP samples were concentrated by ultrafiltration using Amicon Ultra-15 centrifugal filter tubes (Millipore Ltd., 10 K membrane molecular weight cut-off) and washed 4 times with Milli-Q water (4 ml) to remove unbound peptide and residual borohydride.

The NP gold content was determined using a colorimetric gold assay. Hydrodynamic size and zeta potential of the NPs in water/10% PBS were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern instruments). Peptide content of the NP samples was determined by HPLC using a Varian 900-LC, with a reverse phase C18 column (Acquisition time: 11.2 min; Temperature = 35 °C; Slit width = 2 nm; 95% water, 5% acetonitrile gradient (1 ml/min) switching at 8 min, switch to 20% water, 80% acetonitrile). In brief, an aliquot of NPs (10 μl) was mixed with 40 μl of KCN solution (100 mM in 10 mM potassium hydroxide) to dissolve the gold. The sample was analyzed by HPLC at 212 nm to determine released peptide concentration, and at 400 nm to confirm that all the gold was dissolved. An aliquot of NPs (10 μl), diluted with 20 μl TFA, was also analyzed by HPLC to determine whether any free peptide remained in the NP solution, following ultrafiltration purification.

ID injection of peptide-loaded NPs

Mice were anesthetized using isoflurane and the injection site was shaved prior to delivery. Free peptide or peptide-loaded NPs were injected intradermally in 50 μl sterile PBS using a 29G needle.

MN characterization and injection of peptide-loaded NPs

Hollow MN devices (MicronJet600™) were provided by NanoPass Technologies. MicronJet is a CE marked and FDA cleared device, consisting of three MNs, each 600 μm in length, with a lumen of approximately 60 μm in diameter, bonded to a plastic adapter that attaches to a Luer syringe.¹⁴ MicronJet600™

t1.1 Table 1

t1.2 Physicochemical properties of peptides used, listed in order of hydrophobicity with BDC2.5 being the most hydrophobic and OVA being the least hydrophobic (genscript online resource, available at <https://www.genscript.com/ssl-bin/site2/peptidecalculation.cgi>).

t1.3	Peptide	Sequence	Charge	Isoelectric point	pH	% hydrophobic/hydrophilic residues
t1.4	BDC2.5	YVRPLWVRME	1	9.34	Basic	Hydrophobic: 60% Hydrophilic: 10% Neutral: 30%
t1.5	HIP	DLQTLALWSRMD	-1	4.11	Acidic	Hydrophobic: 50% Hydrophilic: 25% Neutral: 25%
t1.6	OTII	ISQAVHAAHAEINEAGR	1	6.5	Basic	Hydrophobic: 47.1% Hydrophilic: 23.5% Neutral: 29.4%

162 injection did not change the diameter of NPs, which remained
 163 physically stable under the shear forces of the injection.²⁶ Mice
 164 were anesthetized using isoflurane, the injection site was shaved,
 165 and peptide was injected in 50 μ l sterile PBS.

166 Adoptive cell transfer

167 Splenic BDC2.5 CD4⁺ T-cells were purified using a MACS
 168 CD4 II negative selection kit (Miltenyi Biotec). Cells were
 169 labeled with 20 μ M CFDA (Invitrogen) in 10% fetal calf serum
 170 (FCS) RPMI at room temperature for 5 min at a concentration of
 171 10^7 /ml. The cells were then washed twice in RPMI containing
 172 FCS, rested for 15 min, and subsequently washed in saline.
 173 These cells were then re-suspended at 4×10^6 cells in 200 μ l
 174 sterile saline and injected intravenously into 6-10-week-old
 175 female mice. At the indicated times, single-cell suspensions were
 176 prepared from spleen and various LNs, and CD4⁺ T-cells were
 177 analyzed by FACS. OTII T-cells were isolated from pooled LNs
 178 and spleen of OTII mice, maintained on a *Rag-2*^{-/-}xB6
 179 [CD45.1] background, using a CD4⁺ T-cell negative isolation
 180 kit (Dynal, Invitrogen). For CTV labeling, purified OTII T-cells
 181 were resuspended in PBS containing 2.5 mM cell tracer violet
 182 (CTV) (Molecular Probes) for 3 min at room temperature. 10^6

CTV-labeled OTII T-cells were adoptively transferred into the 183
 specified mice. 184

Phenotypic analysis by flow cytometry 185

All antibodies were purchased from BioLegend and BD 186
 Pharmingen. 187

A single cell suspension was pre-treated with anti-CD16/32 188
 (BD Pharmingen) and then stained at 4 °C for 25min with the 189
 following pre-titrated mAbs against the indicated antigens: CD4- 190
 AF700 (RM4-5), CD44-BV71 (IM7), CD3e-AF700 (500A2), 191
 CD45.1-APC (A20), CD4-BV786 (RM4-5) and TCR V α 2-PE 192
 (B20.1). To assess intracellular expression, samples were 193
 permeabilized and fixed using Foxp3 transcription factor 194
 staining buffer set (eBioscience), according to the manufacturer's 195
 instructions. Intracellular mAbs against the following proteins 196
 included: CTLA4-BV421 (UC10-4B9), IL10-BV650 (JES5- 197
 16E3) and IFN γ -PeCY7 (XMG-1.2). Cell viability was evalu- 198
 ated using fixable viability dye efluor780 (eBioscience) or 199
 zombie yellow (Biolegend), according to the manufacturer's 200
 instructions. Samples were washed and read using a FACS 201
 LSRII Fortessa cytometer with DIVA software (BD) and 202
 analyzed using FlowJo software (Treestar). 203

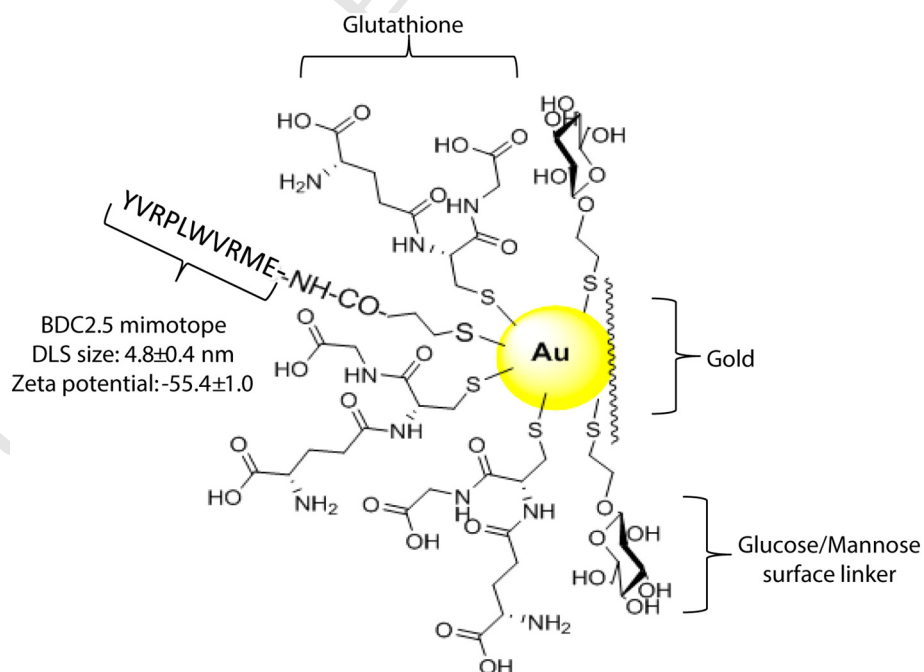


Figure 1. **Gold NP composition.** Composition of gold NPs used, comprising a gold core, conjugated via sulfur bonds to an organic layer of glucose or mannose, glutathione and peptide, (BDC2.5mimotope).

Peptide	DLS size (nm)	Zeta potential	Peptide loading levels
BDC2.5	4.8 ± 0.4	-55.4 ± 1.0	2%
HIP	3.3 ± 1.0	-54.7 ± 1.0	2%
OVA	6.3 ± 0.8	-45.3 ± 0.6	2%

204 Statistical analysis

205 All results are presented as the mean ± standard error of the
 206 mean (SEM) and were analyzed using GraphPad Prism V5.
 207 Student's *t* test, one-way ANOVA and two-way ANOVAs (for
 208 analyses with more than 1 variable) with Bonferroni post-hoc test
 209 were used for statistical analysis. *P* values of <0.05 were
 210 considered statistically significant: *denotes *P* < 0.05, **de-
 211 notes *P* < 0.01, ***denotes *P* < 0.001.

212 Results

213 Pharmacokinetics of intradermally-delivered BDC2.5mimotope 214 peptide coupled to NP differs from BDC2.5mimotope peptide 215 alone in vivo

216 Following the intravenous transfer of CFDA-labeled islet-
 217 specific BDC2.5 CD4+ T-cells into naïve NOD mice,

BDC2.5mimotope NPs, BDC2.5mimotope peptide alone (2 µg 218
 peptide in 50 µl) or peptide free 'blank' NPs were administered 219
 by ID injection at the back of the neck. Lymphoid organs were 220
 then analyzed at 24, 48 and 72 h. Following administration of 221
 BDC2.5mimotope NPs, BDC2.5 T-cell proliferation in the skin- 222
 draining LN (axillary), the non-draining LN (inguinal), the 223
 spleen and pancreatic LN (PLN) was enhanced, compared to 224
 baseline at the 48 and 72 h time points (Figure 2). In contrast, 225
 levels of proliferation were much reduced in mice treated with 226
 BDC2.5mimotope peptide alone; proliferation was only detected 227
 in the axillary (draining) LN at the 72 h time point, with no 228
 proliferation at either 24 or 48 h (and only baseline proliferation 229
 in the PLN) (Figure 2). As expected, modest proliferation of 230
 BDC2.5 CD4+ T-cells as a result of endogenous presentation of 231
 the cognate antigen occurred in the pancreatic LN in control 232
 mice. This was not enhanced following ID injection of peptide 233
 and indicates that the peptide administered alone was not 234
 disseminated to the PLN. No proliferation was observed using 235
 peptide-free 'blank' NPs, confirming the antigen-specific nature 236
 of the response. Overall, ID delivery of BDC2.5mimotope 237
 peptide via NPs resulted in significantly higher levels of T-cell 238
 proliferation in distant lymphoid tissues and at earlier time points 239
 than peptide alone. 240

241 Peptide NP conjugation: the effects of T-cell affinity

242 CD4+ T-cells are central in the process of beta cell 242
 243 destruction. BDC2.5 CD4+ T-cells are highly diabetogenic and 243

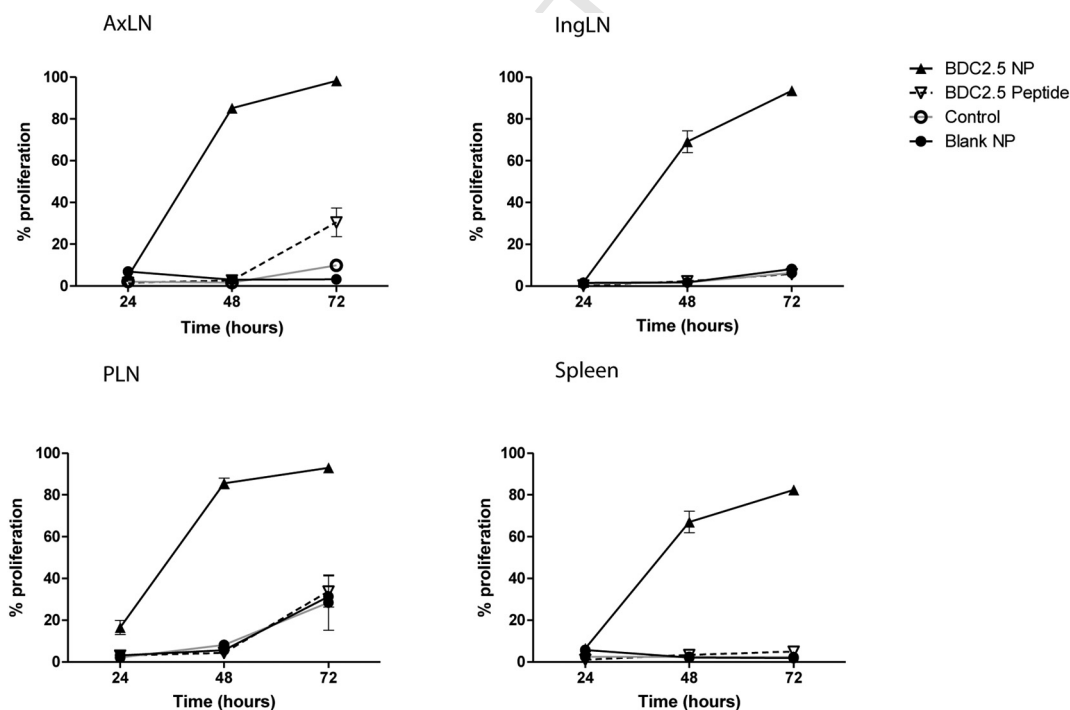


Figure 2. In vivo proliferation time course analysis following peptide-NP delivery compared to peptide alone. CFDA-labeled BDC2.5 CD4+ T-cells were transferred intravenously into NOD mice, followed by an ID injection of either 2 µg BDC2.5mimotope or BDC2.5mimotope-NP or peptide free 'blank' NP. Experimental control mice did not receive the ID injection. LNs (axillary, inguinal and pancreatic) and spleen were harvested at 24, 48 and 72 h following injection and BDC2.5 CD4+ T-cells analyzed for proliferation by CFSE dilution. Mean ± SEM with 3 mice per treatment is shown, representative of 3 experiments. Statistical analysis was done by two-way ANOVA with Bonferroni post-test comparing BDC2.5mimotope NP to BDC2.5mimotope peptide (***) (*P* < 0.001).

244 the natural epitope for these pathogenic cells is a hybrid insulin
 245 peptide (HIP), formed by the fusion of a chromogranin A peptide
 246 sequence WE14 and insulin C-peptide.²² Although naturally
 247 formed, HIP has a lower affinity for BDC2.5 CD4+ T-cells than
 248 the commonly used high affinity islet antigenic peptide substitute
 249 BDC2.5mimotope. Therefore, to determine whether NP conju-
 250 gation changed these relative peptide affinities, NPs were
 251 conjugated to either the high affinity BDC2.5mimotope peptide
 252 or the lower affinity naturally-formed HIP peptide. CFDA-
 253 labeled BDC2.5 CD4+ T-cells were then intravenously trans-

ferred into NOD mice and these mice were subsequently treated 254
 with an ID injection of an NP formulation (BDC2.5mimotope or 255
 HIP peptide or peptide alone. At 72 h, proliferation of transferred 256
 BDC2.5 CD4 T-cells was significantly higher in all lymphoid 257
 tissues following treatment with the BDC2.5 NPs compared to 258
 BDC2.5mimotope peptide (Figure 3, A and B), replicating 259
 earlier findings shown in Figure 2. However, this effect was not 260
 observed using the lower affinity HIP-NPs at an equivalent 261
 peptide dose (2 μ g). Instead, HIP-NPs only induced proliferation 262
 in the axillary LN and at lower levels than those seen with 263

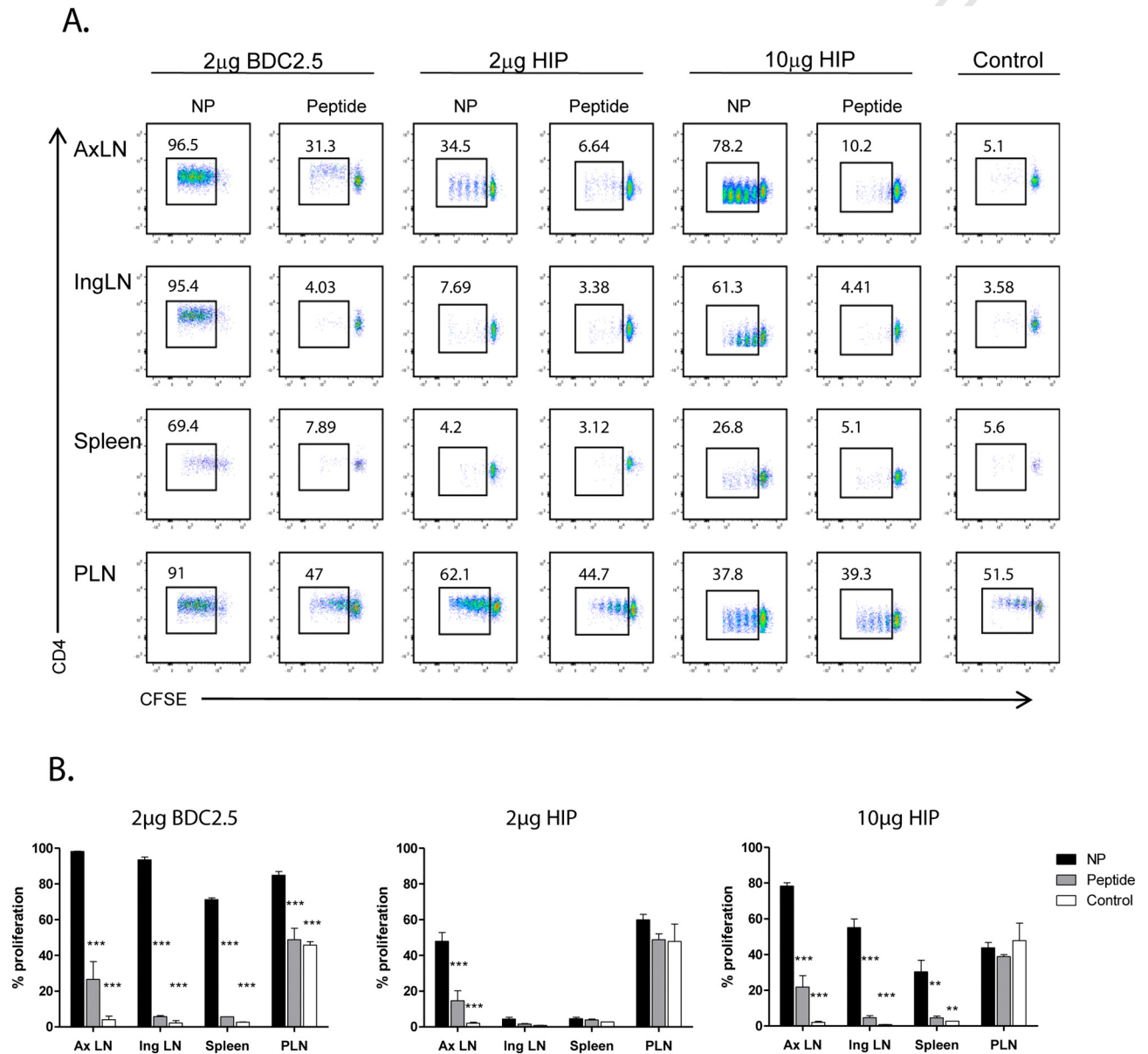


Figure 3. Peptide-NP effects on in vivo T-cell proliferation are dependent on peptide affinity. CFDA-labeled BDC2.5 CD4+ T-cells were transferred intravenously into NOD mice, followed by an ID injection of peptide or peptide-NP. LNs (axillary, inguinal and pancreatic) and spleen were harvested 72 h later and proliferation analyzed by flow cytometry. (A) Representative flow cytometric plots showing proliferative responses from left to right, 2 μ g BDC2.5mimotope-NP, 2 μ g BDC2.5mimotope peptide, 2 μ g HIP-NP, 2 μ g HIP peptide, 10 μ g HIP-NP, 10 μ g HIP peptide and no ID injection. (B) Left to right, summary of proliferation for high affinity BDC2.5mimotope and lower affinity 2 μ g and 10 μ g HIP epitope. Mean \pm SEM of 3 mice per treatment is shown, representative of 3 experiments. Statistical analysis was done using one-way ANOVA with Bonferroni post-test comparing peptide-NP to peptide and control (** $P < 0.01$, *** $P < 0.001$).

264 BDC2.5mimotope-NP, though still significantly higher than HIP
 265 peptide alone. Endogenous proliferation in the pancreatic LN
 266 was not enhanced by 2 µg HIP-NP. No proliferation above
 267 control (no ID injection) was observed in the inguinal LN and
 268 spleen. However, when a higher dose of HIP-NP was
 269 administered (10 µg), widespread proliferation was observed in
 270 these NP-treated mice, with profiles resembling those witnessed
 271 with 2 µg BDC2.5mimotope NP. These results are consistent
 272 with lower amounts of peptide-NP being present in distant LN
 273 and in the case of a lower affinity peptide, may be limiting for T-
 274 cell proliferation at lower doses. Dissemination of peptide alone
 275 was much less efficient than its NP-formulated counterpart and
 276 therefore no presentation was witnessed in the distant LNs, at
 277 any of the doses of HIP peptide that were examined.

278 *Peptide-NP conjugation: effects of peptide solubility*

279 The influence of peptide solubility on peptide-NP behavior
 280 was also evaluated in vivo (Figure 4), by comparing the behavior
 281 of the highly water-soluble OTII peptide, recognized by OTII
 282 CD4+ T-cells, with the less soluble BDC2.5mimotope and HIP
 283 peptide counterparts. CTV-labeled cells were transferred into
 284 C57BL/6 mice, followed by OTII peptide or OTII-NP at 2 µg or
 285 10 µg by ID injection, using 29G insulin needles. At a 2 µg OTII-
 286 NP dose, T-cell proliferation occurred in all the lymphoid organs,
 287 with greatest proliferation in the draining lymph nodes (brachial
 288 and axillary LN). Moreover, at the higher dose of 10 µg, OTII-
 289 NP induced significantly more T-cell proliferation than 2 µg
 290 OTII-NP in the inguinal LN (** $P < 0.01$) and spleen
 291 (** $P < 0.001$). In contrast to both BDC2.5mimotope and HIP
 292 peptide, proliferation of T-cells in response to the OTII peptide
 293 also reached almost 100%.

294 *Peptide-NP can be successfully delivered via MicronJet600™* 295 *microneedles into murine skin*

296 To test whether using MicronJet600™ MNs would enhance
 297 peptide-NP and free peptide delivery in vivo, experiments were
 298 performed in both NOD and C57BL/6 mice. Following
 299 intravenous transfer of BDC2.5 CD4+ T-cells into NOD mice
 300 (Figure 5), and OTII cells into C57BL/6 mice (Figure 6), the
 301 respective mice were injected with 2 µg BDC2.5mimotope-NP
 302 or 2 µg OTII peptide-NP or soluble peptide using MicronJet
 303 MNs. Lymphoid organs were harvested after 72 h and
 304 proliferation was analyzed. Figures 5 and 6 indicate successful
 305 peptide-NPs delivery using MNs, with high levels of prolifer-
 306 ation in the skin-draining brachial and axillary LN, as well as the
 307 inguinal LN and spleen, thus confirming the presentation of the
 308 MN-delivered antigen to the responding T-cells. However, the
 309 use of MicronJet needles, in this context, did not enhance
 310 delivery of the BDC2.5mimotope peptide alone, nor alter the
 311 delivery of OTII peptide compared to conventional ID injection.
 312 Overall, proliferation following MN delivery was comparable to
 313 that observed with conventional ID injection.

314 *Peptide-NP is transported to LN by migratory skin DCs*

315 CCR7, a leukocyte chemotactic receptor, is expressed by
 316 mature DCs and plays a key role in DC homing to LNs, as well as
 317 their subsequent positioning within LN functional compart-

ments. Loss of CCR7 impedes DC migration from non-lymphoid 318
 organs, such as the skin, into the draining LN.^{25,27} With this in 319
 mind, CCR7-deficient mice were used to determine the role of 320
 DCs in distribution of OTII peptide and OTII peptide-NP, and to 321
 determine whether peptide transport is an active DC-mediated 322
 process. ID injection of OTII peptide and OTII-NP was therefore 323
 examined at two different concentrations (2 µg and 10 µg) in wild 324
 type C57BL/6 or CCR7KO C57BL/6 mice. A reduction in 325
 proliferation was observed in different LN of the CCR7KO mice 326
 following administration of the peptide-NP, with a significant 327
 reduction at the higher 10 µg OTII concentration in both the 328
 draining LN and spleen (* $P < 0.05$) (Figure 7). Although the 329
 same trend was observed with 2 µg OTII-NP and peptide alone, 330
 the decrease was not statistically significant. This suggests that 331
 migratory DCs are important for peptide-NP transport from the 332
 skin to the draining LNs, particularly at high concentrations. 333

HIP-NP influences cellular phenotype in a concentration- *dependent manner* 334 335

To determine the impact of HIP-NP concentration on cell 336
 function, NOD mice were transferred with CFDA-labeled 337
 BDC2.5 CD4+ T-cells and then immediately treated with an 338
 ID injection of 0.4 µg, 2 µg, 6 µg or 10 µg HIP-NP or free HIP 339
 peptide. After 72 h, axillary draining LNs, the inguinal LN, 340
 spleen and pancreatic LN were harvested and examined and a 341
 concentration dependent HIP-NP proliferative response was 342
 revealed. Proliferation in the axillary LN, inguinal LN and 343
 spleen, was most notable at 10 µg. This was significantly higher 344
 than the proliferation observed in the HIP peptide-administered 345
 mice (Figure 8, A) and consistent with experiments that were 346
 conducted with a more limited dose range (Figure 3). 347
 Furthermore, the axillary LN T-cells expressed increased levels 348
 of activation markers CD44 and CTLA4 (Figure 8, B), which 349
 reached significance at lower concentrations compared with 350
 proliferation in the inguinal LN and spleen. Despite enhanced 351
 activation status, a significant decrease in cells expressing IFN γ 352
 was noted in the axillary LN of HIP-NP treated mice compared to 353
 peptide alone (* $P < 0.05$). IL-10 expression was low under all 354
 conditions (Figure 8, C). Therefore, while T-cells displayed an 355
 increased activation and proliferation profile over a wide HIP-NP 356
 range, functionally the cells of the draining LN produced less 357
 IFN γ . 358

359 **Discussion**

The lipophilicity of 'naked' peptide autoantigens has been 360
 shown to influence localized delivery in human skin²⁸; results in 361
 murine skin (Figures 2-6) provide further exemplification of this. 362
 The murine dermis is an aqueous region, consisting of 60.3% 363
 water,²⁹ and therefore the notable increase in T-cell proliferation 364
 in response to intradermal administration of the water-soluble 365
 OTII peptide (its cognate antigen) at lymphoid organs distant to 366
 the local environment (Figure 7) is likely to be facilitated by 367
 rapid diffusion in the local environment, uptake by DC in 368
 draining LNs and subsequent distribution in the lymphatic 369
 system. However more lipophilic auto-antigenic peptides such as 370
 BDC2.5mimotope and HIP, which are insoluble in aqueous 371

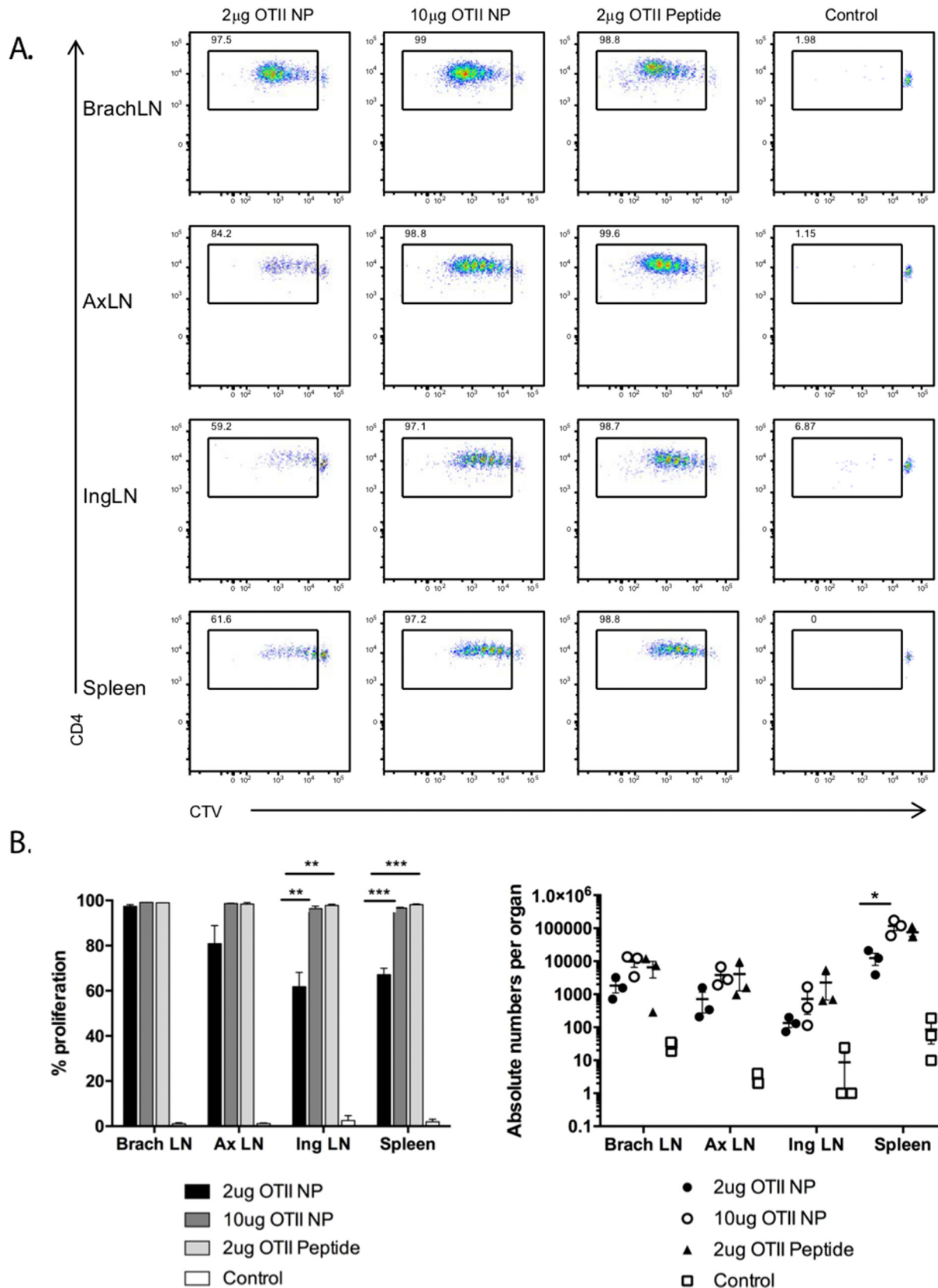
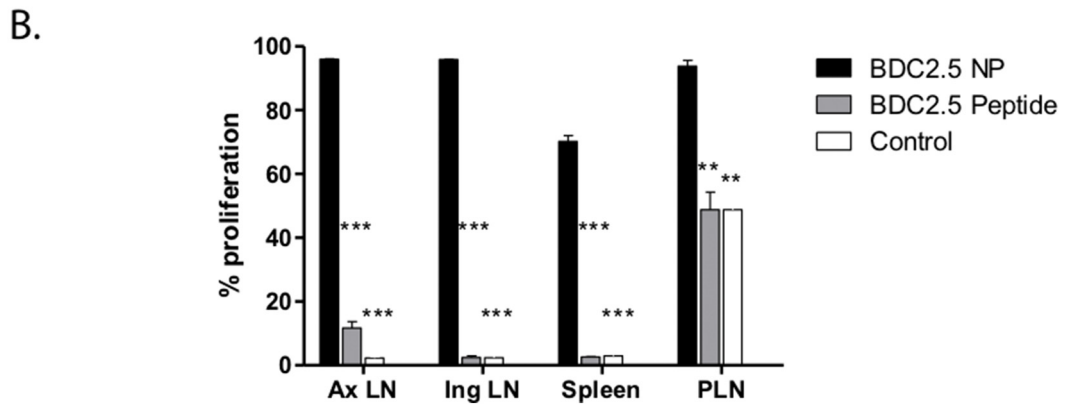
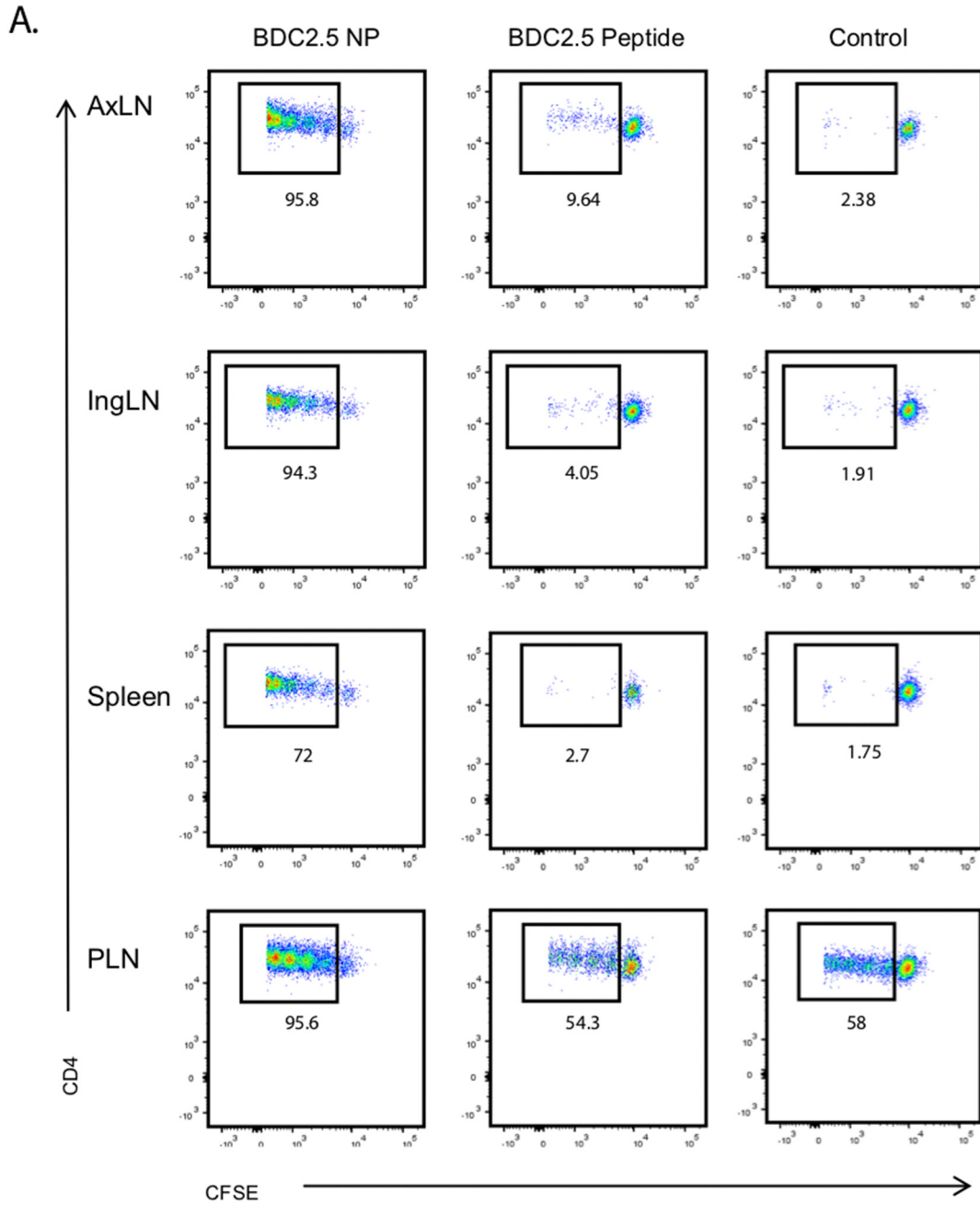
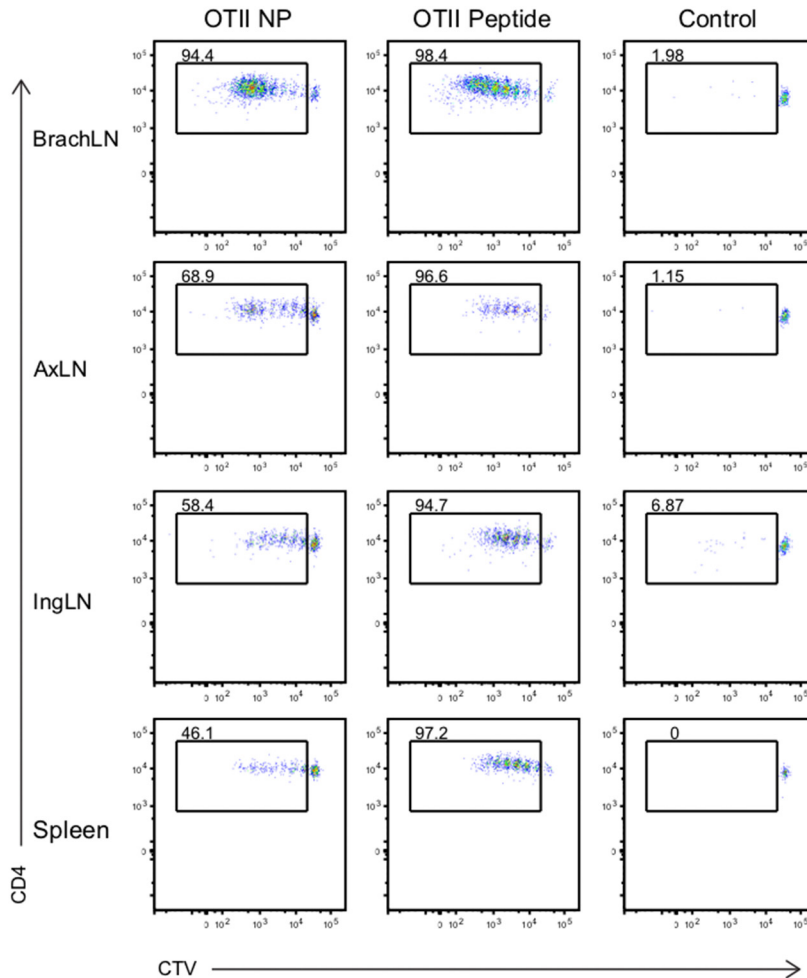


Figure 4. Peptide-NP effects in vivo differ according to peptide solubility. (A) Representative flow cytometric plots showing proliferative responses to (from left to right) 2 µg OTII-NP, 10 µg OTII-NP, 2 µg OTII peptide and controls in the brachial and axillary LNs (draining), distal inguinal lymph node and spleen. (B) Summary of proliferation for OTII-NP and peptide by % proliferation and absolute numbers. Data represent mean ± SEM (% proliferation) and each dot



A.



B.

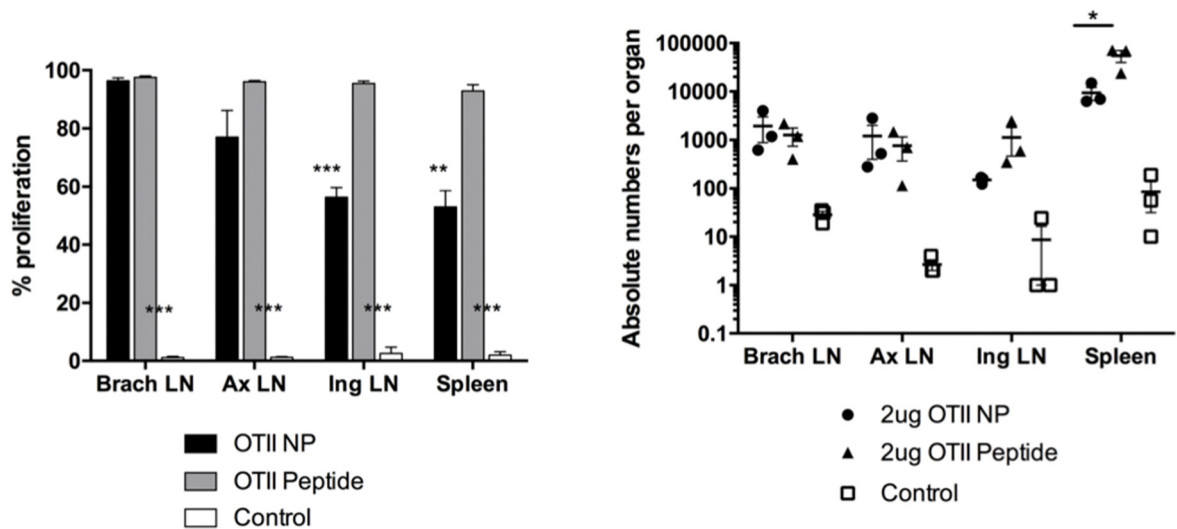


Figure 6. MicronJet600™ delivery of OTII peptide-NP and peptide alone induce proliferation of transferred T-cells. Mice received fluorescently-labeled cells intravenously, followed by an ID injection of peptide or peptide-NP using the short (600 μ m) hollow MN device (MicronJet600™). Lymph nodes were harvested 72 h later and proliferation was analyzed by flow cytometry. (A) Representative flow cytometric plots showing proliferative responses to 2 μ g OTII-NP, 2 μ g OTII peptide and controls in the brachial and axillary lymph nodes (draining), distal inguinal lymph node and spleen following MN delivery. (B) Summary of proliferation for OTII-NP and peptide. Mean \pm SEM (% proliferation) is shown and each dot corresponds to a mouse (absolute numbers per organ). Data are representative of three experiments with $n = 2-3$ animals per group. Significant differences were identified using one-way ANOVA with Bonferroni post-test comparing peptide-NP to peptide and control (** $P < 0.01$ *** $P < 0.001$).

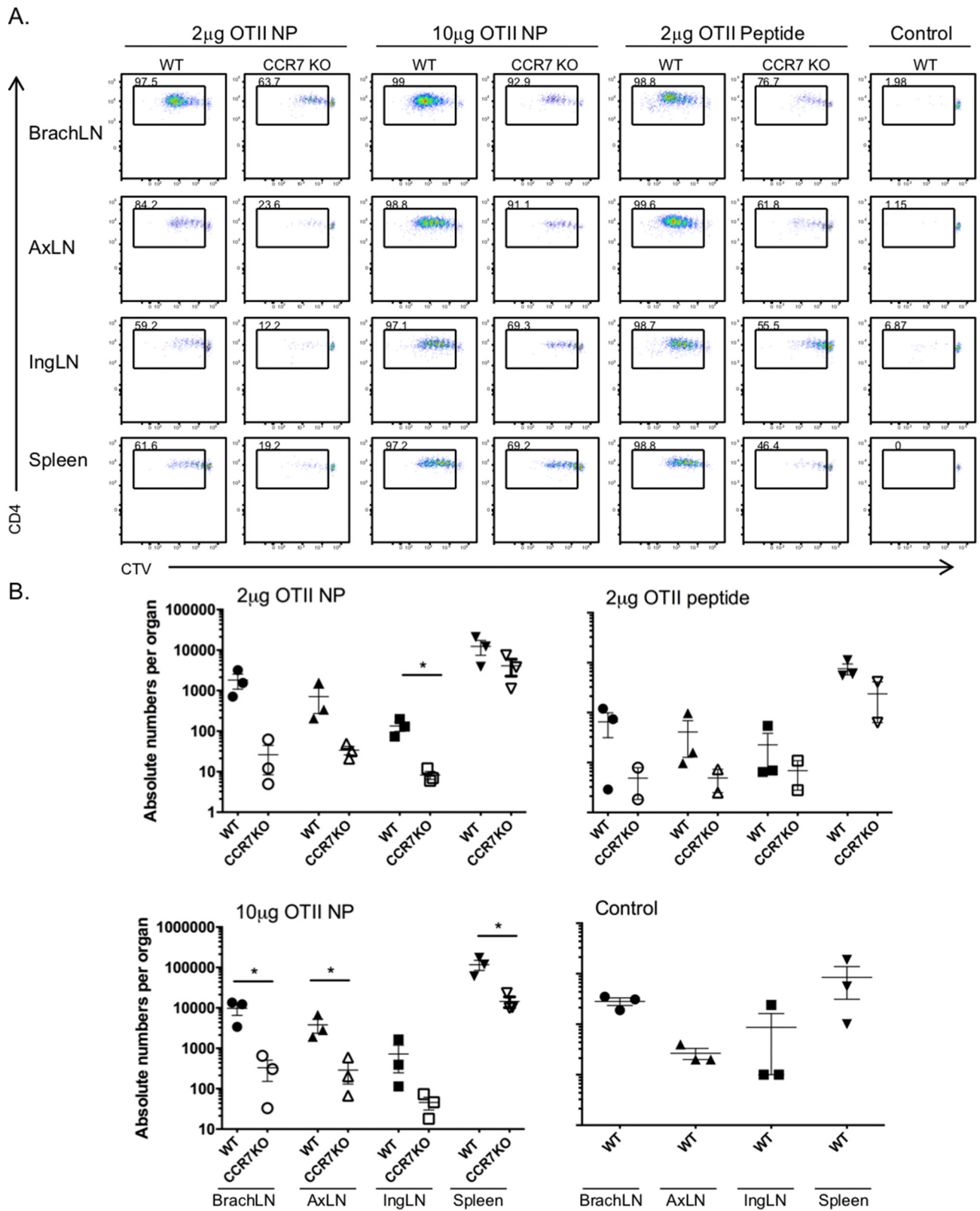


Figure 7. Peptide-NP is taken up by dendritic cells and distributed to lymphoid organs. WT and CCR7KO mice received CTV-labeled OTII T-cells intravenously, followed by an ID injection of peptide or peptide-NP using a 600 μ m MicronJet600™ needle. LNs and spleen were harvested 72 h later and proliferation was analyzed by flow cytometry. (A) Representative flow cytometric plots showing proliferative responses (left to right) to 2 μ g OTII-NP, 10 μ g OTII-NP, 2 μ g OTII peptide and controls in the brachial and axillary LN (draining), distal inguinal LN and spleen. (B) Absolute numbers of proliferating OTII CD4⁺ T-cells in response to OTII-NP and peptide. Each dot corresponds to a mouse and the mean (horizontal bar) is indicated. Data are representative of three

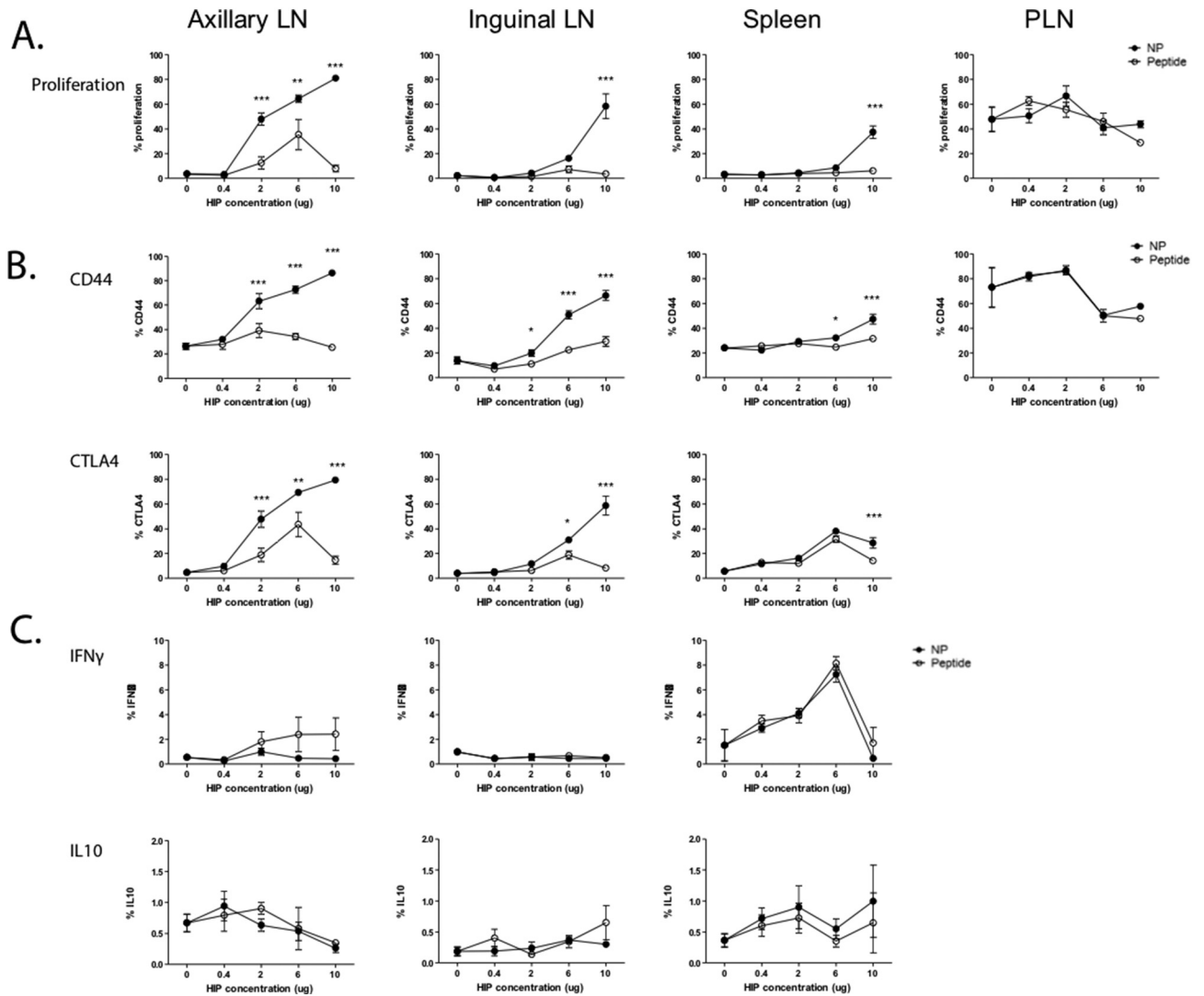


Figure 8. HIP-NP has differential effects on the cellular phenotype dependent on concentration. NOD mice received CFDA-labeled BDC2.5 CD4+ T-cells intravenously, followed immediately by an ID injection (29G insulin needle) of HIP-NP or HIP peptide at various concentrations. LNs were harvested 72 h later and CD4+ T-cells were analyzed by flow cytometry. (A) Proliferative response of cells to HIP-NP or peptide as determined by CFSE dilution. (B) BDC2.5 T-cell activation markers including CD44 and CTLA4 and (C) cytokine response including IFN γ (axillary LN; interaction = ns, NP vs peptide $p = *0.027$, HIP concentration = ns) and IL10. Statistical analysis was done using two-way ANOVA with Bonferroni post-test comparing peptide-NP and peptide at individual concentrations (* $P < 0.5$ ** $P < 0.01$, *** $P < 0.001$). Mean \pm SEM is shown; data are representative of 3 experiments ($n = 3$ per treatment).

372 media, such as water and PBS, are only able to stimulate
 373 response in the local environment i.e. in the draining lymph node
 374 (Figures 2-3). Enhancing the distribution of poorly water-soluble
 375 peptides to stimulate immune responses distant from their
 376 administration site may be desirable for a range of therapeutic
 377 approaches, including immunotherapy for conditions such as
 378 type 1 diabetes.

379 Gold NPs have recently garnered attention as drug delivery
 380 agents for several reasons, including their lack of toxicity,³¹
 381 chemical stability and extensive surface to volume ratio,^{32,33}
 382 which enable conjugation of clinically significant doses of
 383 therapeutics. NPs have previously been used to deliver disease-
 384 relevant autoantigens in mouse models of EAE,³⁴⁻³⁶ arthritis³⁷
 385 ,³⁸ and diabetes.^{18,19,39} Therefore, in this study we have
 386 explored the potential of a gold NP formulation to enhance

387 trafficking of lipophilic peptides from the antigen-presenting cell
 388 (APC) rich skin compartment to distant target sites, such as the
 389 pancreas and pancreatic LN. We have also investigated the
 390 importance of the mechanism of administration (microneedles
 391 versus ID injection), peptide affinity, peptide solubility and
 392 dosing on peptide-NP trafficking in vivo.

393 Peptide-NPs incorporating the relatively insoluble
 394 BDC2.5mimotope and HIP peptides facilitated enhanced
 395 proliferation at LNs distant to the site of intradermal injection,
 396 thus indicating a marked improvement in distribution, in vivo,
 397 compared to peptide alone. Enhanced proliferation of T-cells,
 398 response to the NP-conjugated lipophilic peptides, was detect-
 399 able in the draining LN, the non-draining LN, the spleen and the
 400 PLN, 48 h after intradermal administration. These kinetics are
 401 consistent with a minimum requirement of 24 h for T-cell

proliferation to occur after antigen encounter. Conversely, conjugation of the highly water-soluble OTII peptide to the NP formulation reduced proliferation in the LNs at a “like for like” dose (2 µg) and it was only at an increased dose (10 µg) that the NP formulation performed comparably to the ‘naked’ peptide. This may be explained by a reduction in the diffusive properties of the hydrophilic peptide upon conjugation to a gold NP and, as a corollary, indicates that the enhancement afforded by conjugation of the lipophilic peptides to the ultra-small gold NPs may not be mediated simply by an increase in the solubility and diffusive properties of the therapeutic within the tissue. However, while these results are indicative, the lipophilic and hydrophilic peptides used in this study are detected by two different T-cell clones in two different mouse strains. Future experiments examining a range of peptide solubilities in a single mouse strain are therefore needed to probe this hypothesis further. Further studies could also explore whether NP-conjugation enhances peptide stability in vivo, as this may also contribute to enhanced and prolonged T-cell proliferation.

DCs are abundant in the immunocompetent skin. Increased peptide uptake by DCs in the local environment is more likely to result in increased activation and translocation of these highly effective APCs from the skin to the draining LNs and subsequent presentation to their cognate T-cells. Studies conducted in the CCR7-deficient mouse model at high OTII-NP concentrations (Figure 7) were therefore used to probe the role of DCs in trafficking locally-delivered NP-conjugated lipophilic peptides to distant LNs. Data indicate that distribution to LNs was partly dependent on DCs, with those mice lacking the lymph node homing receptor, CCR7, displaying reduced proliferation in response to peptide-NP administration (Figure 7). Previous studies suggest that intradermally-delivered antigens conjugated to a NP formulation diffuse within the extracellular environment to the draining LN for presentation to resident DCs in a size-dependent manner.^{5,42,43,45,46} Furthermore, it has been hypothesized that ‘large’ NPs, ranging from 33 nm⁴⁵ to 500 nm,⁴⁶ are internalized by DCs at the site of injection and are trafficked to local LNs for presentation, while ‘small’ NPs are not retained in LNs, thereby reducing the magnitude and quality of the ensuing T-cell response^{45,47} and Foxp3 T-reg induction.^{43,48} In contrast to this, we have shown that ultra-small OTII-NPs (2-6 nm) are, in part, taken up by DCs following ID injection and are subsequently transported to the local draining LN. Mice deficient in the LN homing receptor CCR7 demonstrated significantly less proliferation in the draining LN, particularly at the high concentration peptide-NP formulation. We therefore hypothesize that the enhancement in the distribution afforded by gold NP conjugation to poorly soluble peptides is, in part, due to enhanced trafficking from the site of injection by DCs, rather than simply by a change to the diffusive properties of the formulation. This finding is of particular interest, as it implies that peptide-NPs could be formulated with a second tolerogenic cargo that could be targeted and co-delivered to skin DCs (rather than LN resident DCs that may be responsible for presentation of ‘naked’ peptide that reaches the LN by diffusion), thus potentially enabling DC function to be modulated prior to migration to the draining LN.

Different affinities of the peptides to their corresponding antigen-specific T-cells resulted in observable differences in the in vivo response to the BDC2.5mimotope, HIP and OTII peptide-bound NP formulations. BDC2.5mimotope peptide, identified by screening a peptide library,²³ has a high affinity to BDC2.5 CD4+ T-cells whereas HIP, a relatively newly-discovered peptide epitope, has been proposed as the natural peptide for the highly diabetogenic BDC2.5 T-cells and thus, in common with many autoantigens, is relatively lower affinity.²² This relationship was maintained following NP conjugation of the peptides, with HIP-NPs inducing less proliferation than BDC2.5mimotope-NPs at the same peptide dose, thus indicating that NP conjugation to the peptide does not have a detrimental effect on the relative potencies of these auto-antigens.

Previous studies using NPs formulated with an islet autoantigen provide evidence that enhanced expansion of regulatory T-cells in lymphoid organs may translate to a tolerogenic response in diabetes mouse models.^{18,19} This was exemplified by an expansion in T regulatory cells and suppression of diabetes development; however neither study delivered antigen by the intradermal route.^{18,19} In both of these studies the authors reasoned that NPs would also benefit from a second tolerance-inducing cargo. Indeed, sub-cutaneous delivery of antigen-specific NP formulations, intended to induce tolerance in vivo, has typically used additional anti-inflammatory mediators, such as IL10,⁴² rapamycin³⁴ or TGFβ.⁴³ HIP-NP studies were therefore extended to explore whether intradermal delivery and enhanced distribution of the peptide to distant LNs could bring about a phenotypic change that is indicative of tolerance in type 1 diabetes i.e. a reduction in IFNγ, a pro-inflammatory cytokine whose levels correlate with disease progression and which is known to enhance the development of type 1 diabetes in the NOD mouse model.^{40,41} Down-regulation of this pro-inflammatory mediator IFNγ, three days after administration of HIP-NPs (Figure 7), therefore encourages further investigation of HIP-NP as a therapeutic candidate, potentially a pro-tolerogenic addition to the NP formulation to further enhance regulation.

In this study, NPs were administered via an ID injection using both traditional hypodermic needles and hollow MNs (MicronJet600™). In the mouse model, both methods were able to deliver both peptide and peptide NP formulations into the skin and facilitated comparable in vivo responses. While there are well-recognized differences in murine and human skin, here, both methods of delivery proved equal in their ability to deliver peptide and peptide-NP. Both methods are easily translatable to humans, and peptide and peptide-NP are presented in LNs distant to the site of injection.⁹

In conclusion, this study illustrates the potential value of the intradermal delivery of ultra-small gold NPs for enhanced delivery of lipophilic peptide autoantigens to lymphoid organs and the importance of peptide dose, affinity and solubility on distribution to, and T-cell expansion at, these body sites. Peptide-NP formulations therefore potentially provide a valuable means of targeting poorly soluble peptide epitopes to internal directly inaccessible LNs, such as the pancreatic LN, which may be of particular value in type 1 diabetes tolerisation strategies. Future

516 studies will investigate the clinical utility of this drug delivery
517 system in immunotherapy.

Q21 Uncited references

519 30, 44

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<u>Q21</u>	<p>Uncited references: This section comprises references that occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively, delete it. Thank you.</p> <div data-bbox="641 273 1128 388" style="border: 1px solid black; padding: 5px; margin: 10px auto; width: fit-content;"><p>Please check this box if you have no corrections to make to the PDF file. <input type="checkbox"/></p></div>
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