Self-adjuvanting nanoemulsion targeting dendritic cell receptor Clec9A enables antigen-specific immunotherapy

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Non–antigen-specific stimulatory cancer immunotherapies are commonly complicated by off-target effects. Antigen-specific immunotherapy, combining viral tumor antigen or personalized neoepitopes with immune targeting, offers a solution. However, the lack of flexible systems targeting tumor antigens to cross-presenting dendritic cells (DCs) limits clinical development. Although antigen–anti-Clec9A mAb conjugates target cross-presenting DCs, adjuvant must be codelivered for cytotoxic T lymphocyte (CTL) induction. We functionalized tailored nanoemulsions encapsulating tumor antigens to target Clec9A (Clec9A-TNE). Clec9A-TNE encapsulating OVA antigen targeted and activated cross-presenting DCs without additional adjuvant, promoting antigen-specific CD4+ and CD8+ T cell proliferation and CTL and antibody responses. OVA-Clec9A-TNE–induced DC activation required CD4 and CD8 epitopes, CD40, and IFN-α. Clec9A-TNE encapsulating HPV E6/E7 significantly suppressed HPV-associated tumor growth, while E6/E7–CpG did not. Clec9A-TNE loaded with pooled B16-F10 melanoma neoepitopes induced epitope-specific CD4+ and CD8+ T cell responses, permitting selection of immunogenic neoepitopes. Clec9A-TNE encapsulating 6 neoepitopes significantly suppressed B16-F10 melanoma growth in a CD4+ T cell–dependent manner. Thus, cross-presenting DCs targeted with antigen–Clec9A-TNE stimulate therapeutically effective tumor-specific immunity, dependent on T cell help.

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Introduction

Non–antigen-specific stimulatory cancer immunotherapies are commonly complicated by off-target effects. Antigen-specific immunotherapy, combining viral tumor antigen or personalized neoepitopes with immune targeting, offers a solution. However, the lack of flexible systems targeting tumor antigens to cross-presenting dendritic cells (DCs) limits clinical development. Although antigen–anti-Clec9A mAb conjugates target cross-presenting DCs, adjuvant must be codelivered for cytotoxic T lymphocyte (CTL) induction. We functionalized tailored nanoemulsions encapsulating tumor antigens to target Clec9A (Clec9A-TNE). Clec9A-TNE encapsulating OVA antigen targeted and activated cross-presenting DCs without additional adjuvant, promoting antigen-specific CD4+ and CD8+ T cell proliferation and CTL and antibody responses. OVA-Clec9A-TNE–induced DC activation required CD4 and CD8 epitopes, CD40, and IFN-α. Clec9A-TNE encapsulating HPV E6/E7 significantly suppressed HPV-associated tumor growth, while E6/E7-CpG did not. Clec9A-TNE loaded with pooled B16-F10 melanoma neoepitopes induced epitope-specific CD4+ and CD8+ T cell responses, permitting selection of immunogenic neoepitopes. Clec9A-TNE encapsulating 6 neoepitopes significantly suppressed B16-F10 melanoma growth in a CD4+ T cell–dependent manner. Thus, cross-presenting DCs targeted with antigen–Clec9A-TNE stimulate therapeutically effective tumor-specific immunity, dependent on T cell help.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Current approaches to target cross-presenting DCs have chemically or molecularly conjugated antigen to mAbs recognizing specific endocytic receptors (19–22) such as the C-type lectin receptor Clec9A, or DNGR, which is expressed by cross-presenting DCs and plasmacytoid DCs (pDCs) in mice, and CD141+ (BDCA3+) DCs in humans (19, 23, 24). In vivo, Clec9A organizes the processing and cross-presentation of dead cell and viral antigens by MHC class I, resulting in CTL induction for viral and tumor immunity (19, 24). However, Clec9A-antigen conjugates are challenging to manufacture and require additional costimulation or adjuvant to elicit antitumor immune responses. In fact, although Clec9A conjugates reproducibly induce high titers of specific antibody after a single i.v. inoculation without additional adjuvant, CTL induction requires systemic coadministration of agonist anti-CD40 and/or TLR-agonist adjuvant (22, 24), which may increase the risk of systemic inflammatory adverse effects. An alternative approach used a peptide to target Clec9A+ DCs with OVA conjugates but in vivo CTL priming also required adjuvant (25).

Here we combined the concepts of systemic nanoparticle delivery and targeting of antigen to cross-presenting DCs in an alternative approach, in which we encapsulated antigen excipient in a Clec9A-targeting tailorable nanoemulsion (Clec9A-TNE). Oil-in-water nanoemulsion systems have an excellent safety profile and are widely used as clinical vaccine adjuvants (26–30). We reported previously that intraperitoneally injected Clec9A-TNE, grafted with immune-evading polymer PEG and anti-Clec9A mAb using a noncovalent click self-assembly, evaded nonspecific phagocytosis and clearance and targeted antigen to CD8+ DCs in mice. OVA encapsulated within the TNE oil core induced antigen-specific CD8+ T cell responses in vitro (31). Although this platform nanocarrier technology showed that targeting of cross-presenting DCs could be achieved, the immunogenicity of TNE-encapsulated antigen was not explored. Here we show unexpectedly, given the lack of DC activation by anti-Clec9A-antigen conjugates, that in the absence of adjuvant, cross-presenting DCs targeted with antigen-Clec9A-TNE stimulate DC activation, antigen-specific CTLs, and highly effective tumor-specific immunity, dependent on the presence of CD4 helper epitopes and CD40 signaling.

**Results**

To target antigen to Clec9A+ DCs in vivo, we first encapsulated OVA antigen into Clec9A-TNE (OVA-Clec9A-TNE) using a double emulsion method. OVA-Clec9A-TNE had a similar size distribution, as measured by dynamic light scattering, when diluted in water or isotonic PBS (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI96791DS1), and maintained stability when stored at 4°C for up to 256 days (Supplemental Figure 1B). As stability of nanocarriers in physiological conditions is required for effective cellular targeting, the data suggest that OVA-Clec9A-TNE are stable in physiological environments and suitable for i.v. injection. To assess in vivo biodistribution, we labeled Clec9A-TNE and the nontargeting isotype-TNE with the fluorescent lipophilic dye DiR, and injected each preparation i.v. via tail vein into BALB/c mice. Live mice were imaged 24 hours (Figure 1A) and 7 days (Figure 1, B and C) after injection using in vivo imaging. Clec9A-TNE and isotype-TNE but not free dye control accumulated in liver and spleen 24 hours after injection (Figure 1A). At 7 days, Clec9A-TNE accumulated in spleen (Figure 1B), whereas isotype-TNE accumulated in the liver (Figure 1C). Clec9A-TNE but not isotype-TNE were taken up by splenic CD8+ DCs and pDCs. Both TNEs were taken up to a similar extent, small amount by CD8+ DCs (Figure 1D). Rapid antigen degradation in lysosomal compartments reduces cross-presentation but supports MHC class II-mediated presentation, while antigens delivered to early endosomes are cross-presented efficiently (32, 33). To determine the intracellular localization of Clec9A-TNE, we sorted splenic CD11c+ DCs from naive mice, incubated them with fluorescently labeled Clec9A-TNE or isotype-TNE for 3 hours, then stained early endosomes and lysosomes using the cell compartment markers EEA1 and LAMP1, respectively. Internalized Clec9A-TNE colocalized with EEA1 and LAMP1, while isotype-TNE colocalized with LAMP1 only (Figure 1E). These data indicate that, after recognition by the Clec9A receptor, TNEs are internalized and reach early endosomes and lysosomes. In contrast, isotype-TNE are not efficiently taken up by the cells (Figure 1E), but the low levels that are internalized reach only lysosomes.

OVA and other antigens conjugated to anti-Clec9A mAb to target CD8+ DCs have been shown to induce antigen-specific CD4+ and CD8+ T cell proliferation and strong antibody responses in mice, but not DC activation (19). We tested the capacity of OVA-Clec9A-TNE to induce proliferation of antigen-specific T cells and their CTL activity in the absence of adjuvant. CellTrace Violet-labeled (CTV-labeled) CD8+ (OT-I) or CD4+ (OT-II) OVA-specific T cell receptor transgenic T cells were adoptively transferred to B6.SJL-Ptprc mice, followed by i.v. injection of OVA-Clec9A-TNE, OVA-isotype-TNE, empty Clec9A-TNE, or the same quantity of soluble OVA as delivered in TNE. Six days after immunization with OVA-Clec9A-TNE, the proliferative response of transferred antigen-spe-
specific CD4⁺ and CD8⁺ T cells, as determined by dilution in CTV fluorescence intensity, was significantly increased relative to mice immunized with OVA-isotype-TNE or empty Clec9A-TNE or mice injected with the same amount of soluble OVA (Figure 1, F and G). We compared OVA-Clec9A-TNE in the same assay as anti-Clec9A-OVA and isotype-OVA conjugates without or with CpG adjuvant. Only preparations targeting OVA to Clec9A induced OVA-specific CD8⁺ T cell proliferation. The CD8⁺ T cell response was significantly greater in response to OVA-Clec9A-TNE than to Clec9A-OVA conjugate. Clec9A-OVA plus CpG stimulated significantly higher proliferation. Despite this, only OVA-Clec9A-TNE stimulated production of systemic IFN-α, detectable in serum within 24 hours (Figure 1H). OVA257–264 (SIINFEKL) is the dominant OVA CTL epitope in C57BL/6 mice and is commonly used to investigate CTL-mediated killing of target cells. Five days after immunization with OVA-Clec9A-TNE, OVA-isotype-TNE, or soluble OVA, recipients were injected with an equal mix of syngeneic SIINFEKL-pulsed splenic target cells labeled with 5 μM of CTV (CTVhi) and unpulsed syngeneic splenocytes labeled with 0.5 μM of CTV (CTVlo). Twenty hours later, residual SIINFEKL-specific target cells were enumerated relative to unpulsed splenocytes in recipient mice. After immunization with OVA-Clec9A-TNE, approximately 80% of SIINFEKL-specific targets were killed (Figure 1I). In contrast, no OVA-specific killing was induced in mice immunized with nontargeting OVA-isotype-TNE or soluble OVA. Given that Clec9A-TNE traffic to both endosomes and lysosomes and induce CD4⁺ T cell proliferation in vivo, we compared anti-OVA antibody induction by OVA-Clec9A-TNE, OVA-isotype-TNE, and OVA conjugated to anti-Clec9A or isotype mAb (10B4-OVA, GL117-OVA). Each group received an equivalent i.v. dose of 5 μg of OVA. The anti-OVA response induced by Clec9A-OVA conjugate was significantly greater than the response to isotype-OVA, and the response to OVA-Clec9A-TNE was significantly greater than the response to OVA-isotype-TNE and Clec9A-OVA conjugate (Figure 1).
Previously not to activate DCs after i.v. injection (19, 22), suggesting that the TNE delivery system is particularly immunogenic but only when antigen is encapsulated. Furthermore, coadministration of adjuvant — anti-CD40 alone or with poly I:C — with Clec9A-OVA conjugate was required for induction of CTL responses (22, 24). To determine the mechanism of DC activation and CTL induction by OVA-Clec9A-TNE, we first hypothesized that recombinant OVA, which contained sufficient endotoxin to stimulate an NF-κB–GFP reporter cell line (Supplemental Figure 3), activated the TLR4 pathway through ligation of endosomal TLR after Clec9A-mediated nanoparticle uptake and delivery of OVA to the endosome (34), thereby promoting DC activation and CTL induction. To test this, we constructed OVA-Clec9A-TNE using endotoxin-free OVA, and compared CTL induction by OVA-Clec9A-TNE in TLR4−/− and WT littermate control mice. OVA-specific CTLs were induced to a similar extent by OVA-Clec9A-TNE without additional adjuvant and Clec9A-OVA conjugate codelivered with poly I:C in littermate controls (Figure 2B). Thus OVA-Clec9A-TNE induced specific CTLs even when OVA was endotoxin-free. Moreover, OVA-specific CTLs were not significantly changed when OVA-Clec9A-TNE was given the immunogenicity of OVA antigen delivered to DCs when loaded into Clec9A-targeting TNE relative to nontargeting isotype-TNE after i.v. injection in the absence of adjuvant, we determined whether DCs were activated after OVA-Clec9A-TNE administration. OVA-Clec9A-TNE, OVA-isotype-TNE, empty Clec9A-TNE, or isotype-TNE were administered to mice, and 6 hours later splenic DC maturation markers were analyzed by FACS. After injection of OVA-Clec9A-TNE, we observed a surprising increase in CD86, CD80, and CD40 expression by CD8+ DCs, CD8− DCs, and pDCs. OVA-isotype-TNE, empty Clec9A-TNE, and isotype-TNE controls did not affect expression of these DC maturation markers (Figure 2A and Supplemental Figure 2). Given almost exclusive binding of OVA-Clec9A-TNE to CD8+ DCs and pDCs, the upregulation of maturation markers by Clec9A–CD8− DCs suggested that ligation of Clec9A and delivery of OVA may have promoted a systemic cytokine response and secondary DC activation. Furthermore, coadministration of adjuvant — anti-CD40 alone or with poly I:C — with Clec9A-OVA conjugate was required for induction of CTL responses (22, 24). To determine the mechanism of DC activation and CTL induction by OVA-Clec9A-TNE, we first hypothesized that recombinant OVA, which contained sufficient endotoxin to stimulate an NF-κB–GFP reporter cell line (Supplemental Figure 3), activated the TLR4 pathway through ligation of endosomal TLR after Clec9A-mediated nanoparticle uptake and delivery of OVA to the endosome (34), thereby promoting DC activation and CTL induction. To test this, we constructed OVA-Clec9A-TNE using endotoxin-free OVA, and compared CTL induction by OVA-Clec9A-TNE in TLR4−/− and WT littermate control mice. OVA-specific CTLs were induced to a similar extent by OVA-Clec9A-TNE relative to no TNE control. However, OVA-Clec9A-TNE induced specific CTLs even when OVA was endotoxin-free. Moreover, OVA-specific CTLs were not significantly changed when OVA-Clec9A-TNE...
were delivered to TLR4-/- mice (Figure 2B), indicating that OVA-Clec9A-TNE promote induction of CTLs in a TLR4-independent manner. To determine whether MyD88/TRIF-mediated, inflammasome-mediated, or type I IFN-mediated signaling pathways were involved in OVA-Clec9A-TNE–induced DC activation, we screened expression of CD86 by CD8+ cDCs 6 hours after in vivo delivery of OVA-Clec9A-TNE. CD86 induction was impaired in IFNAR-/- and MyD88/Trif –/– but not caspase-1–/– (Casp1–/–) mice relative to WT mice (Figure 2C). Consistent with the IFN dependence of this DC activation, serum IFN-α increased 8-fold relative to untreated WT mice within 6 hours after injection of OVA-Clec9A-TNE (Figure 2D). This rapid burst of IFN-α suggests secretion by activated pDCs, as observed in Figure 2A (35). OVA-Clec9A-TNE did not induce IFN-α when delivered to IFNAR-/- and MyD88/Trif –/– mice, consistent with positive-feedback activation of CD8+ DCs and pDCs by type I IFN, or crosstalk...
between DCs (Figure 2D). These data indicate that delivery of OVA promotes Myd88/TRIF-dependent and type I IFN–mediated DC activation, when targeted to Clec9A+ DCs in the absence of adjuvant. Consistent with a requirement of DC activation for the induction of CTLs, low levels of lytic activity were observed after delivery of OVA-Clec9A-TNE to recipient mice lacking the capacity for inflammasome-mediated, type I IFN–mediated, or Myd88/TRIF-mediated activation (Figure 2E).

CTL induction requires either the activation of DCs presenting a CD8 epitope with adjuvant or costimulatory CD40-mediated crosstalk from CD4+ T cells (17, 36). Since we had observed delivery of OVA-Clec9A-TNE to early and late endosomes in vitro and the induction of both CD4 and CD8 responses in vivo, we explored whether concomitant delivery of CD4 and CD8 epitopes in Clec9A-TNE would be sufficient to activate DCs in vivo. We encapsulated synthetic sterile SIINFEKL OVA 257–264 CD8 epitope, OVA 323–339 CD4 epitope, or both epitopes in Clec9A-TNE. Both OVA-Clec9A-TNE and Clec9A-TNE encapsulating the CD4 and CD8 epitopes, but neither OVA323–339–Clec9A-TNE nor SIINFEKL-Clec9A-TNE, activated DCs (Figure 3A). Furthermore, there was no difference in DC activation by OVA-Clec9A-TNE, Clec9A-TNE encapsulating

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**Figure 5. E6/E7–Clec9A-TNE but not E6/E7–CpG vaccination is immunogenic and controls TC1 tumor growth.** (A) C57BL/6 mice (n = 10) developing tumor after s.c. injection of TC1 were treated with E6/E7-WH-TNE or E6/E7 with 10 μg CpG or left untreated. Mean tumor size is plotted over time for each group, and individual mice are shown. (B) Peripheral blood mononuclear cells were stimulated with HPV16 E7 aa49–57 (RAHYNIVTF) peptide, and IFN-γ production was quantified by ELISPOT. (C) Experimental design as in A; survival curves of tumor-bearing mice are shown. (D) Organs were harvested from untreated, DiR-labeled E6/E7–WH-TNE–treated, or DiR-labeled E6/E7–isotype-TNE–treated TC1 tumor–bearing mice. Images show distribution of DiR-labeled TNE in lung, liver, spleen, inguinal lymph node (ILN), and tumor. *P < 0.05; **P < 0.001; ***P < 0.0001 by 2-way ANOVA test. Survival analyses used the Mantel-Cox log-rank test.
The CD4 and CD8 epitopes, or SIINFEKL-Clec9A-TNE in RAG1−/− mice, which lack T cells (Figure 3A). Clec9A-TNE encapsulating poly I:C or LPS in the absence of antigen activated DCs much less effectively (Supplemental Figure 4). CD4 helper epitopes enhance DC activation though CD154-CD40 interactions. Furthermore, OVA-Clec9A-TNE and Clec9A-TNE encapsulating both CD4 and CD8 epitopes promoted secretion by pDCs of high serum levels of IFN-α (Figure 3B). Consistent with the requirement for CD154-CD40-mediated T cell help, neither DC activation nor OVA-specific CTLs were induced after delivery of OVA-Clec9A-TNE to CD40−/− mice (Figure 3, C and D). When CD11c+ DCs were incubated with OVA-specific CD4+ and CD8+ T cells in the presence or absence of OVA-Clec9A-TNE, IFN-α was expressed only by pDCs (Figure 3E). pDCs secrete a burst of IFN-α in response to CpG DNA sequences, for which signaling of TLR9 in early endosomes is required (37, 38). To test the requirement of TLR9 and CD40L/CD40 signaling for the activation of DCs and IFN-α secretion, we incubated WT, CD40−/−, or TLR9−/− CD11c+ DCs with OVA-specific CD4+ and CD8+ T cells in the presence or absence
These data indicate that Clec9A-TNE are a self-adjuvanting vaccine platform that simultaneously promotes CD4+ and CD8+ T cell responses to delivered antigen, which would be ideal for cancer immunotherapy. To test this, OVA-Clec9A-TNE or OVA-isotype-TNE were administered i.v. once to C57BL/6 mice, 14 days after orthotopic transplant to the mammary fat pad of PyMT-ChOVA breast cancer cells. Six days later, DiR-labeled OVA-Clec9A-TNE were detected in spleen, as expected (Figure 4A). In the primary tumor and lung metastasis, DiR signal colocalized with mCherry, consistent with demonstrated tumor infiltration by CD8+ DCs (39).

Figure 7. Clec9A-TNE encapsulating immunogenic neoepitopes generate antigen-specific T cell responses and suppress the growth of B16-F10 tumors. (A) C57BL/6 mice (n = 7) were inoculated s.c. with B16-F10, then treated with WH-TNE or G-actin-TNE loaded with a pool of B16-F10 mutated epitopes, as described in Methods, or left untreated. Mean tumor size is plotted over time for each group. (B) C57BL/6 mice (n = 10) were inoculated s.c. with B16-F10 and treated with WH-TNE or G-actin-TNE loaded with a pool of B16-F10 mutated epitopes or left untreated 8 days after tumor inoculation. Survival curves of tumor-bearing mice are shown. (C) Peripheral blood mononuclear cells were stimulated with B16 epitopes or endogenous survivin peptide, and IFN-γ production was measured by ELISPOT. (D) Tumor growth (± CD4-depleting antibody) in mice inoculated s.c. with B16-F10 and treated with WH-TNE loaded with the pool of B16-F10 mutated epitopes or left untreated (n = 7). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by 2-way ANOVA test. Survival analyses used the Mantel-Cox log-rank test.

of OVA-Clec9A-TNE. The OVA-Clec9A-TNE promoted increased CD40L expression by OVA-specific CD4+ T cells and IFN-α secretion into the supernatant in a CD40- and TLR9-dependent manner (Figure 3, F and G). Taken together, our data indicate that after Clec9A-targeted OVA antigen delivery, CD8+ DCs are activated as a result of type I IFN release and antigen-specific CD40-CD40L-mediated CD4+ T cell help, to induce antigen-specific CTLs without additional adjuvant. Activation of IFN-α secretion requires CD40 signaling as well as TLR9. The latter is most likely activated as the TNEs reach the early endosome (37, 38).
Tumor growth was significantly inhibited in mice treated either with OVA-Clec9A-TNE or with OVA-isotype-TNE, but inhibition was greater with OVA-Clec9A-TNE (Figure 4B). The clinical effect of OVA-Clec9A-TNE was associated with significantly greater infiltration by CD11c+F4/80+CD206– inflammatory DCs and CD4+ and CD8+ T cells (Figure 4, C and D, and Supplemental Figure 5). In the AT3-OVA model of breast cancer, survival of mice was significantly greater after a single administration of OVA-Clec9A-TNE than Clec9A-OVA or isotype-OVA conjugates with or without CpG adjuvant, or OVA-isotype-TNE (each mouse received 5 μg OVA) (Figure 4E). These data indicate that OVA-Clec9A-TNE are taken up within tumors, promote an inflammatory tumor environment, and prolong the survival of tumor-bearing mice.

Since clinical translation may be hampered by immunogenicity of the Clec9A mAb, we exploited the highly conserved F-actin component of the cellular cytoskeleton and a recently described WH peptide as specific Clec9A ligands to functionalize nanoparticles targeting either mouse or human cross-presenting DCs (25, 40). OVA-WH-TNE had a similar size distribution to OVA-Clec9A-TNE, and maintained stability in serum conditions when stored at 4°C for up to 25 days (Supplemental Figure 1C). In vivo uptake of F-actin–TNE or WH-TNE by mouse CD8+ DCs and pDCs was similar to that of Clec9A-TNE, while control G-actin–TNE were not taken up (Figure 4F). Specificity was confirmed by the lack of binding of Clec9A-TNE, F-actin–TNE, or WH-TNE to CD8+ DCs (Supplemental Figure 6). Moreover, mice immunized with F-actin–TNE, or WH-TNE loaded with OVA, generated antigen-specific proliferative and cytotoxic responses similar to those of OVA-Clec9A-TNE (Supplemental Figure 7).

To test the efficacy and immunogenicity of Clec9A-TNE for the oncogenic human papillomavirus 16 (HPV16) antigen E6/E7, we generated WH-TNE encapsulating recombinant HPV16 E6/E7 protein and commenced weekly immunization 14 days after s.c. implantation of TC1 tumors. While weekly E6/E7 and CpG adjuvant had no significant impact on tumor growth, WH-E6/E7–TNE significantly suppressed TC1 tumor growth (Figure 5A), induced strong E6/E7–specific IFN-γ responses in peripheral blood 21 and 35 days after tumor implantation (Figure 5B), and significantly increased survival of tumor-bearing mice (Figure 5C). After tumors became established, we injected DiR-labeled WH–E6/E7–TNE i.v. to assess biodistribution. Consistent with evidence that CD8+ DCs infiltrate tumors (39) and that Clec9A-TNE promote an inflammatory tumor environment (Figure 4, C and D), WH-Clec9A-TNE but not G-actin–TNE localized to tumors as well as spleen and liver, while isotype-TNE localized only to spleen and liver (Figure 5D). These data indicate that Clec9A-TNE effectively target antigen to tumor-infiltrating DCs.

While oncopgenic virus expression generates predictable immune target antigens suitable for tumor vaccines, neoepitopes derived from somatic tumor mutations constitute an alternative source of vaccine antigens. In order to exploit peptide neoepitopes for vaccination of the murine B16-F10 melanoma model, we first developed a system to rank immunogenicity of individual neoepitopes using WH-TNE. B16-F10 melanoma cells were implanted s.c., and mice were immunized 15 days later with WH-TNE loaded with a pool of 18 previously described C57BL/6 B16 neoepitopes (41). After 10 days, splenocytes were harvested and restimulated with individual neoepitopes, to compare IL-10 (Figure 6, A and B) and IFN-γ (Figure 6C) production by CD4+ T cells and expression of CD107 by CD8+ and CD4+ T cells (Figure 6, D and E) as a marker of degranulation. Regulatory epitopes were identified as low stimulators of IFN-γ and CD107 and high stimulators of IL-10, and were discarded. The resultant stimulatory pool, consisting of 6 neoepitopes (five CD4 neoepitopes, MUT20, MUT25, MUT30, MUT36, and MUT44, and one CD8 neoepitope, MUT33, with highest immunogenicity), was encapsulated into WH-TNE. Two days after s.c. implantation of B16-F10 melanoma, mice were immunized with WH or control G-actin TNE encapsulating the 6-neoepitope pool. WH-neoepitope-TNE but not G-actin-neoepitope–TNE significantly suppressed tumor growth and enhanced survival (Figure 7, A and B) and induced strong IFN-γ immunity to pooled B16 epitopes and to the universal tumor antigen, survivin (ref. 42 and Figure 7C). Immunogenicity and antitumor effects of the WH-neoepitope-TNE vaccine were Th cell–dependent, as the significant tumor suppression observed with WH-TNE was lost if CD4+ T cells were depleted during treatment (Figure 7D). These data indicate that immunization with WH-neoepitope-TNE stimulates epitope-specific and additional tumor antigen–specific immunity, as well as tumor suppression, in a CD4+ T cell–dependent manner. Notably, in the same experiment, all tumor-free mice (n = 5) previously treated with WH-neoepitope-TNE were protected by subsequent challenge with B16-F10 cells, consistent with the induction of a memory response.

Discussion

The Clec9A receptor has been shown to mediate efficient delivery of antigens derived from necrotic cells to cross-presenting DCs. Virally infected cells undergo lysis, thereby exposing the Clec9A ligand F-actin. CD8+ DCs take up antigen from dying cells via Clec9A, then shuttle F-actin-bound viral proteins into the cross-presentation pathway (43). We show here that Clec9A-TNE are a highly efficient and immunogenic system for targeted antigen delivery to early endosomes and lysosomes in cross-presenting DCs, which can be exploited for generation of CTLs and cognate T cell help using protein antigen or pooled CD4 and CD8 tumor neoepitope peptides. Accordingly, CTLs and high titers of antibody were induced after a single systemic administration of OVA-Clec9A-TNE. In contrast, when delivered as a conjugate, anti-Clec9A-antigen efficiently induced specific antibody, but CTLs were not induced without concomitant adjuvant or anti-CD40 delivery, commensurate with the lack of effect of the conjugate on DCs in vivo (19, 24). In contrast, we found that a single administration of antigen–Clec9A-TNE activated DCs and induced secretion of IFN-α and specific CTLs very efficiently without additional adjuvant in vivo in a manner dependent on presentation of CD4 and CD8 cognate epitopes, the presence of CD4+ T cells, and expression of CD40, MyD88, and IFNAR. Our data are consistent with a model in which, after Clec9A-targeted antigen delivery, CD8+ DCs are sufficiently activated as a result of pDC type I IFN release and antigen-specific CD40-CD40L-mediated CD4+ T cell help to induce antigen-specific CTLs without additional adjuvant. Of particular interest, CD8+ tumor-infiltrating DCs are also targeted by antigen–Clec9A-TNE, leading to local immune acti-
viation, including greater infiltration by inflammatory DCs than myeloid suppressor cells and CD4+ and CD8+ T cell infiltration. Furthermore, in contrast to passive DC-targeting strategies (7, 44–46), a single administration of OVA-Clec9A-TNE inhibited the growth of and enhanced survival with the relatively immunogenic PyMT-OVA and AT3-OVA breast cancer models. After vaccine administration, the burst of IFN-α provoked systemic activation of CD8+ and CD8- DCs. IFN-α is a critical predictor of immunogenic presentation of antigen and antitumor responses after anthracycline chemotherapy and RNA lipoplexes (7, 47). Indeed, stimulation of IFN-α secretion emerged as the key difference between targeting Clec9A as an OVA conjugate or functionalized on TNE. Crosstalk between DCs is essential in vivo for efficient CTL induction. While pDCs cross-present weakly, if at all, they are known to produce large amounts of IFN-α when signaled through TLR9 and CD40, which in turn activates CD8+ and CD8- DCs for efficient antigen presentation (48, 49). Based on previous observations, we speculate that TLR9 activation is triggered as the TNEs reach the early endosome (37, 38).

Our findings indicate that the Clec9A-targeting vaccination platform can efficiently exploit the immunogenicity of the unique epitopes expressed by oncogenic viruses or generated by somatic mutations, which represent the most potent antigens for cancer immunotherapy. Indeed, HPV16 synthetic long peptides used within conventional vaccination approaches induced strong antitumor immunity and clinical responses in patients with HPV16-driven carcinomas (50–52). More recently, neoantigen vaccines in the form of RNA polyepitopes or based on the use of synthetic long peptides on previous observations, we speculate that TLR9 activation is activated when the TNEs reach the early endosome (37, 38).

Preparation of protein antigen in oil dispersion and TNE. Protein antigen solution (10 mg/ml) was prepared by dissolving of protein in ultrapure water. Cithrol GMO HP solution (1%, wt/vol) was prepared by dissolving of Cithrol GMO HP in hexane. Protein and Cithrol GMO HP solutions were mixed in a glass vial using a sonicator for 1 minute at 20 W to form a stable water-in-oil emulsion. The emulsion was frozen rapidly in dry ice before lyophilization. The antigen–Cithrol GMO HP pellet was dissolved in Miglyol 812 to 5 mg/ml and used as oil phase. Lyophilized AM1 was dissolved in HEPES containing ZnCl2 and then homogenized using an ultrasonicator. TNEs were added to PEylated DAMP4 solution, then vigorously stirred (P20-TNE). mAb-DAMP4 was added to P20-TNE and stirred; then Ab-P20-TNE was

Methods

Materials and mice. AM1 (molar mass 2,473, 95% purity) (56), WH peptide (WPRFHSVFTHTGGGK), 4T1, and B16-F10 mutated epitopes (>95% purity) were synthesized by GL Biochem. Miglyol 812 was purchased from AXO Industry SA; CellTrace Violet (CTV) from Molecular Probes; albumin from chicken egg white (OVA) from Sigma-Aldrich; EndoGrade Ovalbumin (98% purity, <1 EU/mg) from Hyglos GmbH; mPEG-NHS (MW 5,000, protein dispersibility index <1.08, purity >95%) from Nanocs; and FITC–anti-CD3 (clone 17A2), APC/Cy7–anti-CD8 (clone 53.67), PE/Cy7–anti-CD11c (clone N418), APC-anti-CD317 (clone 927), PE-anti-CD45.2 (clone 104), PerCP/Cy5.5–anti-F-A1/E (clone M5/114.15.2), PerCP/Cy5.5–anti-CD40 (clone 3/23), PE-anti-CD80 (clone 16-10A1), FITC-anti-CD86 (clone GL-1), APC-anti-CD4 (clone RM4-5), PerCP/Cy5.5–anti-CD45 (clone 13/2.3), PE/Cy7–anti-CD44 (clone 1L7), PE-anti-CD1 (clone 29F.1A12), APC/Cy7–anti-CD4 (clone RM4-5), APC/Cy7–anti-CD11b (clone M1/70), FITC–anti-F4/80 (clone BM8), and APC-anti-CD206 (clone C068C2) from Biologend. Anti–mouse LAMP1 (clone 1D4B) and anti–mouse EEA1 (clone 14/EEAI) were from BD Biosciences. LIVE/DEAD Fixable Aqua Dead Cell Stain was from Molecular Probes. Cithrol GMO HP was a gift from Creda Europe Ltd. DAMP4 fused with antibody (mAb-DAMP4) was generated as previously described (19, 57). CpG ODN 1668 (catalog tlrl-1668) was purchased from InvivoGen, and administered at 5 nmol per dose. Mice were purchased from the Animal Research Centre or bred at the University of Queensland, QIMR Berghofer, or James Cook University under specific pathogen–free conditions.

Versatile, personalized, antigen-specific cancer vaccines are a long-sought therapeutic strategy in cancer immunotherapy. Clec9A TNE represent such a platform to deliver recombinant tumor protein or neoepitope antigens specifically to cross-presenting DCs. This platform can fully exploit the neoepitope target repertoire for personalized immunotherapeutic approaches.
Samples were acquired on a Beckman Coulter CytoFLEX. Using the FoxP3 transcription factor staining buffer set (eBioscience), intracellular staining, samples were fixed, permeabilized, and stained with CD11c, CD206, CD4, and CD8 surface markers at 4°C for 40 minutes. Thereafter cells were stained for CD45, CD11b, F4/80, and CD16/CD32 blocking antibodies (440 U/ml; BD Biosciences) for 15 minutes and then incubated with rat anti–mouse Fc III/II receptor (CD16/CD32) blocking antibodies (440 U/ml; BD Biosciences) for 15 minutes. After 40 seconds at 37°C in a shaking bath, the cell suspension was washed intensively before staining and flow cytometry analysis. Secretion of IFN-α for 16 hours. Cells were washed intensively before staining and flow cytometry analysis. Secretion of IFN-α for 16 hours. Cells were washed intensively before staining and flow cytometry analysis.

For confocal microscopy, splenic CD11c⁺ DCs purified from C57BL/6 mice were plated on coverslips and incubated with Dil-labeled Clec9A-TNE or isotype-TNE for 1 hour at 37°C. While washing to remove unbound TNE, cells were incubated in media overnight. Cells were fixed with 4% paraformaldehyde and permeabilized with 100 μg/ml digitonin (Invitrogen) and stained with rat anti–mouse LAMP1 or rat anti–mouse EEA1, then Alexa Fluor 488–secondary antibody and DAPI (Molecular Probes). Mounted glass coverslips were imaged on an Apotome microscope (Carl Zeiss).

In vitro DC stimulation with TNE. Splenic DC11c⁺ DCs were purified from C57BL/6, CD40⁻/⁻, or TLR9⁻/⁻ mice (58, 59). CD8⁺ and CD4⁺ T cells purified from lymph nodes and spleens of OT-I and OT-II transgenic mice by negative selection were labeled with CTV. CTV-labeled T cells (2 × 10⁴) were cultured with 10⁵ DCs, with or without 2 μl TNE, for 16 hours. Cells were washed intensively before staining and flow cytometry analysis. Secretion of IFN-α in supernatants was assessed by ELISA (VeriKine Mouse IFN Alpha ELISA Kit).

In vivo proliferation assays of transgenic T cells. B6.SJL-Ptprc a mice were inoculated orthotopically with 10⁵ AT3-OVA or 1.5 × 10⁵ PyMT-mChOVA cells into the mammary gland. For therapeutic experiments in the melanoma model, C57BL/6 mice were inoculated s.c. with 2.5 × 10⁴ B16-F10 melanoma cells into the flank and randomly distributed into treatment groups. For therapeutic experiments in the HPV-related cancer model, 10¹ TCI cells were inoculated s.c. into the flank of C57BL/6 mice. Tumor volumes were measured with calipers and calculated using the formula (A × B²)/2 (A as the largest and B as the smallest diameter of the tumor). In therapeutic experiments in the PyMt-mChOVA model, mice received 1 dose of OVA-Clec9A-TNE or OVA-isotype-TNE (5 μg of OVA per mouse i.v.) either 8 or 14 days after tumor inoculation. In therapeutic experiments in the B16-F10 model, mice received 4 weekly i.v. doses of G-actin-TNE or WH-actin-TNE encapsulating E6/E7 protein or a pool of 6 neopeptopes, respectively (10 μg of mutated epitopes per mouse), commencing 2 days after tumor inoculation. Peptides used in B16-F10 studies included FRKAKFLHWYGTEADEMTFEAASN (MUT20), STANYNTHSLNNDIVQFENPVWK (MUT25), PSDKPSQEFVDENVSPVLDSTQFP (MUT30), DSGSPFPAAVLRLDHMGRLKLYHQ (MUT33), CGTAFSDFINFAHHSARIPFVGM (MUT36), EFKHIKADRTFANPQPMVFATPGM (MUT44), and ATFKNWVPFL (survivin20–22, universal peptide).

Expression and purification of E6/E7 fusion protein. E6/E7 fusion protein was prepared as previously described (61). In brief, E. coli BL21(DE3) cells transformed with vector encoding E6/E7 fusion protein were used to express the recombinant protein. LB plates containing kanamycin sulfate were streaked, and a single colony was selected and inoculated into LB media containing kanamycin, and expanded. Protein expression was induced with isoprplyl-thio-galactopyranoside (IPTG). Cell pellets were stored at -80°C until further use. Proteins were purified by Ni-NTA agarose (Qiagen Ni-NTA Fast Start Kit). Endotoxin was eliminated using Pierce High Capacity Endotoxin Removal Spin Columns. Protein concentration was measured by a Thermo Fisher Scientific NanoDrop ND-1000 spectrophotometer before encapsulation into TNE.
Splenocyte stimulation and CD107/IFN-γ/IL-10 analysis. Spleens were removed from tumor-bearing mice and mechanically disrupted through a 70-μm cell strainer into a single-cell suspension. Erythrocytes were lysed by ACK lysis buffer. Splenocytes (10^6 cells per 200 μl per well) were seeded into a 96-well plate and cultured with or without individual epitopes (10 μg/ml) at 37°C for 8 hours. BV241-anti-mouse CD107α (1D4B, Biolegend) and BV241-anti-CD107b (ABL-93, BD Biosciences) were added to evaluate degranulation of cytotoxic granules. T cells producing IL-10 and IFN-γ were detected by intracellular cytokine staining following addition of GolgiPlug (catalog 555029, BD Biosciences) during the last 5 hours of stimulation. Staining was performed using FoxP3/Transcription Factor Staining Buffer Set (catalog 00-5523-00, eBioscience).

ELISPOT. The IFN-γ release enzyme-linked Immunospot (ELISPOT) (41) was carried out using monocytoids isolated by a 2-hour plastic adherence step from naïve syngeneic splenocytes as antigen-presenting cells and CD3+ peripheral blood T lymphocytes from tumor-bearing mice using the Pan T Cell Isolation Kit II (Miltenyi Biotec) as responders. Effector and responder cells (25,000 each) were cultured at 37°C for 48 hours in the presence of anti-IFN-γ-coated (10 μg/ml, clone AN18) Multiscreen 96-well plates (Millipore). Cytokine secretion was detected with an anti-IFN-γ antibody (1 μg/ml, clone R4-6A2) stimulated without or with 10 μg/ml of peptide. All samples were tested in triplicate.

Statistics. Results are presented as mean with SD of each group. Data were statistically analyzed by ordinary 1-way or, where appropriate, 2-way ANOVA and Tukey’s multiple-comparisons test, using GraphPad Prism software. P values of 0.05 were considered significant. Survival analyses used the Mantel-Cox log-rank test.

Study approval. The animal experiments were approved by Animal Ethics Committees of the University of Queensland, Brisbane, Australia, and James Cook University, Townsville, Australia.

Author contributions
BZ, APJM, RM, IC, ML, RD, and RT designed the study. BZ, MT, DM, and KMT conducted and analyzed experiments. AG, KM, AGB, IC, and ML provided reagents. BZ, IC, RM, ML, RD, and RT wrote the manuscript. All authors read and approved the final draft of the manuscript.

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