INTRODUCTION

There are currently two main influenza vaccines available to the public. The tri- or tetravalent inactivated seasonal influenza vaccines (IIV) that are administered intramuscularly, and that consist of two influenza A strains (H1 and H3) and one or two influenza B strains, and the live attenuated influenza vaccine (LAIV) that is given intranasally. While the LAIV vaccine has proven efficacious in children,\(^1\text{-}^3\) efficacy in adults aged >65 has been considerably lower.\(^4\text{-}^5\) Consequently, the IIV vaccines are most commonly used in adults. There are, however, other challenges with the use of live attenuated vaccines, such as the possibility of the virus reverting to a more pathogenic strain,\(^6\) in addition to the LAIV vaccines being unsuited for immunizing people with certain immune deficiencies.\(^7\)

The use of subunit vaccines is a safe alternative to attenuated vaccines, but these vaccines have been troubled...
by low immunogenicity. However, targeting subunit vaccines to dendritic cells (DCs) by conjugating antigen to chemokines or antibodies is a well-established method for enhancing immunogenicity, and has been shown to efficiently enhance both T cell and antibody responses.8-10 Since the initial description of DCs as a separate cell population in 1973,11 it has become apparent that there are several different subsets of MHC-II+CD11c+ DCs in both lymphoid (spleen and lymph nodes) and non-lymphoid tissues (skin, intestine, liver, lung and skeletal muscles) which differ in terms of functions and specializations.12 Lymphoid-resident and tissue-resident conventional DC type 1 (cDC1) have been shown to selectively express the Xcr1 receptor,13,14 and to excel at cross-presentation of exogenous antigen on MHC class I molecules to CD8+ T cells.15,16 The other conventional DC subset—cDC2, can be identified based on expression of Sirp1 or CD11b and have been reported to be more efficient at presenting peptides on MHC-II to CD4+ T cells.17,18

Due to their role in the induction of CD8+ T cell responses, several receptors have been evaluated for delivery of antigen to cDC1s, including DEC205, Clec9a and Xcr1.19-24 We and others have observed that targeting antigen to the Xcr1 receptor by fusion to an anti-Xcr1 antibody or the chemokine Xcl1, the ligand of Xcr1, induces strong CD8+ T cell responses in mice.22,23,25 In addition, DNA vaccination with Xcl1-fusion vaccines in BALB/c mouse induces a highly IgG2a-dominated antibody response, indicative of Th1 polarization.22,26,27 In this study, we investigate the effect of targeting haemagglutinin (HA) from influenza A to Xcr1+ cDC1s in the lung by intranasal immunization. Our observations indicate that Xcl1-HA fusion vaccine enhances induction of antigen-specific T cell responses in lung and spleen, and provide full protection against a lethal challenge with influenza virus.

2 | MATERIALS AND METHODS

2.1 | Cell lines, antibodies and viruses

HEK293E and MDCK cells were maintained in RPMI or DMEM, respectively, supplemented with 40 mg/mL Gensumycin (Sanofi-Aventis Norge AS), 50 µM monothioglycerol (Sigma), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Lonza). HEK293E cells were used for transient transfection and expression of HA-fusion proteins and Xcl1-mCherry protein. Stably transfected N50 cells were used to express mCherry protein. The cell lines were purchased from ATCC.28 For serum IgG ELISA, anti-mouse IgG (Fc-specific), anti-mouse IgG1-bio (clone 10.9), anti-mouse IgG2a-bio (clone 8.3), anti-IgA (α-chain specific, goat polyclonal) were used. For flow cytometric analysis, Anti-CD3e-FITC (145-2C11, Tonbo Biosciences), anti-CD19-FITC (1D3, Tonbo Biosciences), anti-CD49b-FITC (DX5, eBioscience), anti-Ly6G-FITC (1A8, Tonbo Biosciences), CD45R/B220-FITC (RA3-6B2, Tonbo Biosciences), anti-MHC-II-A700 (M5/114.15.2, BioLegend), anti-CD11c-APC (N418, Tonbo Biosciences), anti-CD11b-PerCP-Cy5.5 (M1/70, Tonbo Biosciences), anti-CD24-BV510 (M1/69, BioLegend), anti-CD103-PerCP-Cy5.5 (M5/114.15.2, BioLegend), anti-CD3e-pacific blue (17A2, Tonbo Biosciences) and anti-IFNγ-APC (XMG1.2, Tonbo Biosciences) were used. For challenge experiments, influenza strain A/Puerto Rico/8/34 (PR8, H1N1) and A/California/07/09 (H1N1pdm09) were used.

2.2 | Animals

All animal experiments were approved and performed according to the Norwegian National Animal Research Authority (NARA). Female BALB/c mice aged 6-8 weeks were obtained from Janvier Labs, France and housed under minimal disease conditions. For challenge experiments with influenza virus, mice were euthanized if they lost more than 20% of their initial body weight as a humane clinical endpoint, according to the guidelines of NARA.

Mice were challenged 2 weeks after the last immunization by i.n. inoculation with 5 × LD50 A/Puerto Rico/8/34(H1N1) or A/California/07/09 diluted in PBS, 10 µL into each nostril after i.p. sedation with a ZRF-cocktail containing Zoletil Forte 250 mg/mL Virbac France, Rompun 20 mg/mL, Bayer Animal Health GmbH and Fentanyl 50 µg/mL, Actavis, Germany, 0.1 mg/10 g body weight.

2.3 | Intranasal immunization

For the determination of vaccine dose, mice were vaccinated once by intranasal delivery of 10, 3, 1, 0.3 or 0.1 µg mXcl1-mCherry protein with 50 µg of Poly (I:C) (HMV) VacciGrade™ Invivogen, or 25 µg of CpG ODN 1585 VacciGrade™ Invivogen, diluted in PBS. 10 µL of diluted vaccine were administered into each nostril, total volume of 20 µL pr mouse, under sedation by intraperitoneal (i.p) injection of ZRF-cocktail. As a negative control, mice were vaccinated with 50 µg Poly I:C in PBS or 25 µg of CpG in PBS.

For mice vaccinated with Xcl1-HA, αNIP-HA or HA, 3 µg or 1 µg of protein were used with 50 µg of Poly I:C in PBS, to a total amount of 35 µL, or 17.5 µL into each nostril, under the same condition as mice vaccinated with mCherry containing fusion molecules described above.
2.4 | Generation and purification Xcl1 fusion vaccines

The fusion vaccines consist of two monomers coupled into a dimeric molecule originated from the construct plNOH2.²⁹ It consists of (i) a human dimerization unit consisting of hinge 1, hinge 4 and Cγ3 from human γδ, (ii) an antigenic unit, either mCherry or haemagglutinin (HA) from the influenza strain A/H1N1/Puerto Rico/8/34, and (iii) the chemokine Xcl1 as a targeting unit.²² As a non-targeted control, αNIP that contains a single-chain variable fragment (scFv) specific for the synthetic hapten 4-hydroxy-3-ido-5-nitrophenylacetic acid was used.²⁹ As a monomeric antigen control, purified haemagglutinin with a his-tag was used.

Plasmids encoding Xcl1-mCherry, Xcl1-HA, αNIP-mCherry and αNIP-HA fusion vaccines were transiently transfected in a five-layer multi-flasks (BD Falcon™) using Lipofectamine 2000 (Invitrogen) as a transfection reagent. Supernatants were harvested after 4-5 days, and applied onto an affinity column containing CaptureSelect™ FcXL Affinity Matrix (Life Technology) specific for the dimerization domain present in the fusion vaccines. The column was coupled to an Äktaprimer Plus (GE Healthcare Biosciences AB). Bound fusion vaccines were washed with PBS and eluted in 0.1 M Glycine-HCl pH 2.7 and dialysed against PBS. The protein concentration was measured by DeNovix DS11+ spectrophotometer (Thermo Scientific) by calculating the proteins molecular weight (MW) and A280 Molar Extinction Coefficient. As an extra control to determine the protein concentration proteins were analysed by Pierce BCA Protein Assay Kit, (Thermo Scientific).

Haemagglutinin protein was produced using cDNA encoding with a C-terminal his-tag. As for fusion proteins, HEK293E cells were transiently transfected using plasmid DNA but with polyethyleneimine-MAX (PEI-Max; MW 4000, Polysciences) as transfection agent. Cells were grown in T175 bottles and supernatants were harvested every second day for up to 12 days after transfection. His-HA protein was purified using a HisTrap HP Column (GE Healthcare) coupled to a BioLogic workstation and a BIO-RAD recorder. Bound protein was eluted with 250 mM imidazole in PBS, pH 7.2-7.4 and monomeric his-HA protein were isolated using a Superdex 200 Increase 10/300 GL column (GE Healthcare) followed by determining protein concentration using DeNovix DS11+ spectrophotometer.

2.5 | Coomassie blue staining

Purified proteins were analysed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoreses) using a Bolt™ 4%-12% Bis-Tris Plus gel, 1.0 mm x 12 well (Invitrogen by Thermo Fisher Scientific). For non-reducing proteins, 4 µg of each protein, 6× staining buffer containing 12% SDS, 300 mM Tris pH 6.8% and 0.05% bromphenolblue were diluted in PBS to a total volume of 20 µL. For reducing samples, 2 µL of dithiothreitol (DTT) (Sigma) was included. Samples were heated at 95°C for 5 minutes before being loaded onto the gel. A PowerEase 300 W was used as a power supply, 200 V, 3.00 Ampere for 35 minutes, and gels were stained in Coomassie stain (0.1% Brilliant Blue R250 in 10% acetic acid, 50% methanol, and 40% H2O) for 15-20 minutes on shake after heating. Gels were then destained in destaining solution (10% acetic acid, 40% methanol, and 50% H2O) with at least two changes for 30-60 minutes on shake and stored in dH2O.

2.6 | Binding of Xcl1-mCherry fusion vaccines to lung DCs

For ex vivo staining, single-cell suspensions of lung tissues from BALB/c mice were generated using gentleMACS™ C tubes according to manufactures protocol for lung tissues. 1 × 10⁶ cells were stained with anti-CD3ε, anti-CD11b, anti-CD11c, anti-CD14, anti-CD19, anti-CD24, anti-CD49b, anti-CD103, anti-MHC II and anti-ly6G, in addition to Xcl1-mCherry or αNIP-mCherry and >5 × 10⁵ cells analysed on BD LSR Fortessa.

For in vivo staining, BALB/c mice received a single-intranasal immunization of 10 µg Xcl1-mCherry or αNIP-mCherry. After 48 hours, mice were sacrificed and lungs were removed after perfusing five mL of sterile PBS into the left heart ventricle for removal of circulating blood. Next, single-cell suspensions of lung tissue were generated and stained and analysed as described above, with exception of adding the Xcl1-mCherry fusion proteins to the staining mix.

2.7 | ELISPOT

Single-cell suspensions from spleens were used for ELISPOTPLUS mouse IFNγ kit (Mabtech AB). Briefly, 1 week after intranasal vaccination spleens were harvested and dissociated in a gentleMACSTM C tubes with MACS® Tissue Dissociation Kit according to manufactures protocol for spleen tissue and incubated for 30 minutes at 37°C. Cells were passed through a 70-µm cell strainer (Fisherbrand, Fisher Scientific). After Tris-Buffered Ammonium Chloride (ACT) treatment for 5 minutes, cells were washed with PBS and counted in Countess™ (Invitrogen™). Cells were then seeded at 5 × 10⁵ cells in 200-µL RPMI (with supplements) per well in duplicates and stimulated with either IYSTVASSL 2 µg/mL
(MHC-I restricted peptide), HNTNGVTAACSHEG and SVSSFERFEIFPK (MHC-II restricted peptide) or irrelevant peptide (Ag85 M tuberculosis) for 18 hours at 37°C in 5% CO₂. Next, cells were incubated with detection antibodies according to manufactures protocol. For counting of spots, CTL-Immune® S6 Micro Analyzer, CTL Europe GmbH, were used.

### 2.8 Intracellular IFNγ staining of lungs or spleens

Spleen and lung tissue were harvested 7 days after intranasal vaccination as previously described and single-cell suspensions were obtained as described for ELISPOT assay. Lung or spleen cells were seeded at 1 × 10⁶ cells in 100 μL RPMI (with supplements) in a 96-well plate and stimulated with the MHC-I restricted peptide IYSTVASSL (2 μg/mL), MHC-II restricted peptide HNTNGVTAACSHEG and SVSSFERFEIFPK (2 μg/mL), a negative control peptide or mCherry protein (2 μg/mL) at 37°C for 22 hours. The last 4 hours of stimulation BD Golgistop™ were added. Cells were subsequently stained with anti-CD3, anti-CD4, anti-CD8, anti-CD19 and anti-CD14. Cells were protected from light and stored on ice during extracellular staining. After a quick fixation with 3% of Paraformaldehyde, cells were intracellular stained with anti-IFNγ, washed and >5 × 10⁵ cells analysed per sample on a BD LSR Fortessa.

### 2.9 Serum ELISA

Blood sampling of mice was performed by puncturing vena saphena 14, 28, 42 and/or 64 days after immunization. Blood samples were left to coagulate at RT for approximately 30 minutes before centrifugation at top speed for 10 minutes in a Hereaus Pico 17 Centrifuge (Thermo Scientific). Sera were collected and stored at −20°C until analyse.

High binding 96 wells ELISA plates (Costar nr: 3590; Corning) were used and coated with either purified mCherry protein (1 μg/mL), inactivated PR8 influenza virus (2 μg/mL) (Charles River Laboratories) or recombinant murine Xcl1 (2 μg/mL, R&D Systems) and incubated overnight at 4°C. The following day, plates were blocked with PBS/BSA for a minimum 1 hour at room temperature (RT). After a washing step, serum samples were diluted in 1:50 from individual mice to each well and subsequently serially diluted 1:3 in ELISA buffer and incubated overnight at 4°C. Next, plates were washed and incubated for 2 hours at RT with anti-mouse IgG (Fc-specific), anti-mouse IgG1-bio (clone 10.9) or anti-mouse IgG2a-bio (clone8.3) (BDPharmingen). After washing, plates incubated with anti-IgG1 or anti-IgG2a were also incubated with streptavidin-ALP (GE Healthcare [RPN1234V], 1:3000) for 45 minutes at RT. ELISAs were developed by adding substrate buffer (1 mg/mL phosphate substrate; Sigma), and plates were measured at a wavelength of OD405 after 25 or 50 minutes on a Tecan Sunrise spectrophotometer (Tecan Group Ltd.). Antibody titre was defined as the highest dilution of serum sample with OD values > (mean +3 × SD) of PBS vaccinated mice. If OD values did not exceed that of the given PBS vaccinated mice (mean +3 × SD), the sample was given an end point titre of 1.

### 2.10 ELISA for detecting IgA in bronchoalveolar lavage

Bronchoalveolar lavage was harvested by inserting a 18G peripheral venous catheter into the trachea of the immunized mice, and injecting and aspirating 1 mL PBS into the lung. The samples were frozen at −20°C until analysis. To evaluate titres of IgA, high binding 96-well ELISA plates were coated with purified mCherry protein (1 μg/mL) and incubated ON at 4°C. The IgA ELISA was performed as described above for serum IgG ELISA, with the exception that BAL was diluted 1:10 in the top well, and subsequently serial dilution 1:3. IgA was detected using an AP-conjugated anti-IgA (α-chain specific).

### 2.11 ELISA for detecting HA fusion proteins

High binding 96 wells ELISA plates (Costar nr: 3590; Corning) were used and coated with mouse anti-human IgG CH3 domain, 2 μg/mL (MCA878G; AbD Serotech) and incubated overnight at 4°C. The following day, plates were blocked with blocking buffer as earlier described and 1 μg/mL of protein were added in triplicates and serially diluted 1:3 in ELISA Buffer. Plates were stored at 4°C overnight. Next, after washing, plates were incubated with anti-HA antibody, H36-4-52, a kind gift from Siegfried Weiss, for 2 hours at RT. Plates were washed and incubated with Streptavidin alkaline phosphatase, 1:3000, GE Healthcare, for 45 minutes at RT. ELISAs were developed and analysed as described above.

### 2.12 Neutralization assay

Neutralization was assessed as previously published. In short, serum from immunized mice was diluted 1:3 with receptor destroying enzyme (RDE), and incubated at 37°C.
overnight before the serum was inactivated by incubation at 56°C for 30 minutes. 30 µL inactivated serum was added to the top well of a 96-well cell culture plate and diluted with 70 µL virus diluent (40 mL DMEM, with 1 mL 1 M Hepes, 4.64 mL 10% albumin fraction V [in H2O], 45 µL TPCK-Trypsin [1 mg/mL]), and 50 µL virus diluent was added to the remaining wells. The serum was serially diluted 2-fold by transferring 50 µL from the top row to the next row, mixing and transferring 50 µL to the third row. After transferring to the final row and mixing, 50 µL was discarded. Subsequently, 50 µL of virus diluent containing 100xTCID50 A/Puerto Rico/8/34 was added to each well, and the serum/virus mix incubated at 37°C for 2 hours. Next, MDCK cells were added at a concentration of 20 000 cells in 100 µL virus diluent to each well, and the plates incubated ON at 37°C and 5% CO2. The next day, the plates were fixed by removing the serum, washing once with PBS and incubated ON at 37°C for 2 hours. After washing 3x in wash buffer (0.3% Tween 20 in PBS), and then incubating for 1 hour at RT with anti-nucleoprotein (H16-L10-4R5 biotin ab, 0.7 µg/mL). The plates were then washed and incubated with streptavidin alkaline phosphatase (1:3000) for 1 hour at RT. Finally, the plates were washed and developed as described for serum ELISA.

2.13 | Statistics

All statistical analyses were performed using the PRISM 8.0 software (GRAPHPAD Software). Significant differences in antibody titres were determined using Kruskal-Wallis test with Dunn’s multiple comparison test due to the non-continuous nature of the data, while significant differences in the T cell data were calculated using one-way-ANOVA with Tukey’s multiple comparison test (*P < .05, **P < .01, ***P < .001). Differences in weight curves after infection were calculated using two-way ANOVA (*P < .05, **P < .01, ***P < .001). Differences in survival were calculated by Mantel-Cox (*P < .05, **P < .01, ***P < .001).

3 | RESULTS

3.1 Xcl1-targeted fusion vaccines specifically bind cDC1 in lung

Previous work from our laboratory has led to the development of a dimeric vaccine molecule consisting of a targeting unit, a dimerization unit and an antigenic unit (Figure 1A).29,31 Using the chemokine Xcl1 as a targeting unit we have been able to target antigens specifically to Xcr1+ cDC1 in spleen and skin.25,26,32 However, for an influenza vaccine, intranasal delivery may be more relevant, as this is the natural route of infection. To evaluate the possibility of mucosal delivery, and more specifically targeting antigens to Xcr1+ cDC1s in the lung, we first incubated single-cell suspensions of lungs from BALB/c mice with Xcl1-mCherry fusion proteins. As a negative control, the previously published non-targeted anti-NIP-mCherry (referred to as αNIP) was included.26 Specific binding was evaluated by flow cytometry where DCs were defined as lin−MHC-Ⅱ+CD11c+ cells and divided into cDC1 and cDC2 based on the expression of CD24 and CD11b (Figure 1B, Figure S1A). In addition, lung cells were stained with anti-CD103 as previous studies have indicated that Xcr1 is expressed at a higher level on CD103+ cDC1s, compared to CD103− cDC1s.14 As expected, we observed that Xcl1-mCherry specifically bound lung cDC1, but not cDC2 (Figure 1C). Among the cDC1 population, Xcl1-mCherry predominantly bound CD103+ cells, although a slight increase in binding to the CD103+ population could also be observed (Figure 1C). To evaluate the ability of Xcl1-fusion proteins to target Xcr1+ DCs in vivo, mice were immunized intranasally (i.n.) with 10 µg of Xcl1-mCherry and lungs harvested after 24 hours. As controls, mice immunized with αNIP-mCherry or PBS were included. As observed with the ex vivo staining, Xcl1-mCherry was preferentially taken up by CD103+ cDC1, and not CD11b+ cDC2 (Figure 1D).

3.2 | Intranasal immunization with Xcl1-targeted fusion vaccines induce strong antibody responses

Based on the observation that Xcl1-targeted fusion vaccines bound Xcr1+ DC in lung after i.n. delivery, we set out to evaluate the antigen-specific immune responses with this vaccination strategy. We therefore generated fusion vaccines containing HA from influenza A/PR/8/34 as an antigen.22,33 Xcl1-HA and αNIP-HA fusion vaccines were purified, and tested for size and quality by SDS-PAGE (Figure S1B). From the gel, it appears that the concentration of αNIP-HA was higher than Xcl1-HA. However, when performing ELISAs on the purified proteins, Xcl1-HA and αNIP-HA gave near-identical dilution curves, suggesting comparable concentrations (Figure S1C). To determine the optimal vaccine regimen for our assays, BALB/c mice were immunized with 10, 3, 1, 0.3 or 0.1 µg Xcl1-mCherry in the presence of 25 µg CpG or 50 µg poly(I:C) (Figure S2). Xcl1-mCherry was used for these titration experiments due to scarcity of the HA-fusion vaccines. Administration of Xcl1-mCherry formulated with CpG or poly(I:C) induced strong antigen-specific IgG
responses, and maintained the preferential induction of IgG2a previously seen after DNA vaccination. While the highest antibody titres were observed after i.n. immunization with 10 µg Xcl1-mCherry, the results were not significantly different from 3 µg Xcl1-mCherry with 50 µg poly(I:C). Consequently, i.n. immunization with 3 µg protein with the addition of 50 µg poly(I:C) was chosen for the subsequent immunization experiment. We did not detect any significant levels of IgA after one dose of 3 µg Xcl1-mCherry with the addition of 50 µg poly(I:C) (data not shown). However, we did see antigen-specific IgA in BAL on week 6 if the mice were boosted with a second dose after 4 weeks (Figure S2C). We nevertheless chose to continue with one immunization, due to the strong IgG responses observed.

To evaluate immune responses induced by Xcl1-HA, BALB/c mice were immunized once with 3 µg Xcl1-HA, αNIP-HA or HA in combination with 50 µg poly(I:C) and serum samples harvested 14 days after immunization (Figure 2). Both Xcl1-HA and αNIP-HA immunized mice induced strong HA-specific IgG responses, and no significant difference was observed in the magnitude of the IgG response (Figure 2A). There was also no significant difference between Xcl1-HA and αNIP-HA in the induction of HA-specific IgG1 or IgG2a, and consequently the IgG2a/IgG1 ratio, which is in contrast to our previous studies where intradermal DNA immunization with Xcl1-HA induced an antibody response dominated by the IgG2a subclass (Figure 2B-D). There was a tendency for a higher IgG1 titres with αNIP-HA, but the difference was not significant (Figure 2B). Both Xcl1-HA and αNIP-HA did, however, induce significantly higher antibody responses compared to HA (Figure 2A-C). Somewhat surprisingly, we did not observe induction of HA-specific antibodies with 3 µg HA with poly(I:C). In contrast, use of a commercially available HA trimer induced strong antibody responses, suggesting that the lack of immunogenicity is related to monomeric structure of HA generated in the laboratory (Figure S3A). These results further suggest that the bivalent structure of the fusion vaccine construct is beneficial for induction of antibody responses, as the HA3s in the fusion vaccines are also present as monomers. We did not detect any antibodies against Xcl1 in the immunized mice, indicating that the combination of Xcl1-HA and poly(I:C) did not break tolerance towards the chemokine (Figure S3B).

The observed antibody responses were maintained at similar levels for both Xcl1-HA and αNIP-HA immunized mice for at least 8 weeks, suggesting induction of long-lasting antibody responses (Figure 2E). To evaluate if the induced antibody responses could neutralize influenza
virus, we performed a micro-neutralization assay on serum samples harvested 8 weeks after vaccination with Xcl1-HA or αNIP-HA. While the IC50 titres were relatively low, we observed a significant increase in neutralizing antibodies in serum from mice immunized with Xcl1-HA compared to PBS (Figure 2F). There was also an increase in neutralizing antibodies in serum from αNIP-HA immunized mice, but the difference did not reach significance (Figure 2F).

3.3 | Intranasal immunization with Xcl1-HA enhances T cell responses

A key rationale for targeting antigen to cDC1 has been to induce stronger CD8+ T cell responses, as seen previously after intradermal DNA vaccination or laserporation. To test if this effect was maintained when antigen was delivered i.n., BALB/c mice were immunized with 3 μg Xcl1-HA, αNIP-HA or HA formulated with 50 μg poly(I:C). Spleens were harvested after 7 days and induction of IFNγ secreting T cells evaluated by ELISPOT.
Interestingly, Xcl1-HA induced significantly higher numbers of IFNγ secreting cells, compared to the non-targeted αNIP-HA and monomeric HA, after stimulation with both MHC-I and MHC-II restricted peptides (Figure 3A-B). As T cell responses in the lung play an important role in clearing the viral infection, we also analysed lungs harvested from immunized mice. Single-cell suspensions from lungs were stimulated with MHC-I and MHC-II restricted peptides, and evaluated for IFNγ secreting cells by intracellular flow cytometry. Similar to the ELISPOT data, Xcl1-HA induced higher numbers of IFNγ^+CD4^+ and IFNγ^+CD8^+ T cells in lung after immunization compared to the non-targeted vaccines (Figure 3C-D, Figure S4). We also observed significantly more IFNγ^+CD4^+ T cells with HA compared to αNIP-HA, demonstrating that the monomeric HA protein was indeed immunogenic despite not inducing antibody responses. In conclusion, our results indicate that targeting antigen to cDC1 in the lung enhance levels of T cells in the lung, as well as systemic T cell responses.

### 3.4 Intranasal immunization with Xcl1-HA +poly(I:C) induce full protection against a lethal influenza infection

To evaluate whether the induced immune responses seen with Xcl1-HA were sufficient to mediate protection against influenza infection, BALB/c mice were immunized i.n. with 3 µg Xcl1-HA, αNIP-HA or HA with 50 µg poly(I:C) and subsequently challenged with 5xLD50 influenza A/Puerto Rico/8/34 (Mt. Sinai sub-strain) (H1N1). Weight loss was monitored after infection as a sign of disease progression, and mice euthanized if they lost more than 20% weight. Mice immunized with Xcl1-HA or αNIP-HA displayed a slight dip in weight that was most prominent on day 6, and then quickly regained their weight. In contrast, mice immunized with HA or PBS quickly lost weight and had to be euthanized by day 8 after infection (Figure 4A-B). As both Xcl1-HA and αNIP-HA mice survived challenge equally well we suspect antibodies rather than T cells might have conferred the observed protection.

Previous experiments have demonstrated that laser-assisted intradermal delivery of Xcl1-OVA induces antigen-specific immune responses in the absence of adjuvant. To test if the addition of adjuvant was necessary when delivering HA fusion vaccines i.n., mice were immunized with Xcl1-HA or αNIP-HA in the presence or absence of 50 µg poly(I:C). Serum samples were harvested after 2 weeks and tested for the presence of HA-specific IgG (Figure 4C). In contrast to dermal delivery, i.n. immunization with Xcl1-HA or αNIP-HA in the absence of adjuvant did not result in induction of HA-specific antibodies. In accordance with this observation, mice immunized without adjuvant quickly lost weight after challenge with 5xLD50 PR8 virus and had to be euthanized (Figure 4D).

While antibodies against HA tend to be strain-specific, T cell responses against conserved epitopes can provide heterologous protection against related influenza strains. To test this possibility, mice were immunized once with 3 µg Xcl1-HA or αNIP-HA with 50 µg poly(I:C), where HA was derived from A/Puerto Rico/4/34, and subsequently challenged with 5xLD50 influenza A/California/07/09 (H1N1pdm09) after 2 weeks. However, Xcl1-HA immunized mice were not protected after challenge, suggesting that the induced T cells were not sufficient to mediate heterologous protection (Figure 4E).

In summary, our observations demonstrate that i.n. immunization with Xcl1-HA vaccine with poly(I:C) enhances T cell responses, induces strong antibody responses and full protection against homologous influenza infection. Since the expression of Xcr1 is conserved in mammals, this vaccine strategy can potentially be translated to human and veterinary medicine.

### 4 DISCUSSION

In this study, we have evaluated intranasal delivery of a cDC1 targeted influenza vaccine consisting of HA fused to the chemokine Xcl1 in a dimeric fusion vaccine format. Our observations indicate that targeting cDC1 enhances HA-specific T cell responses in spleen and lung. In addition, Xcl1-HA induces strong antibody responses, although targeting did not enhance antibody titres compared to a non-targeted αNIP-HA control. Due to the important role of antibodies in mediating protection in the murine influenza challenge model, both Xcl1-HA and αNIP-HA mediated full protection against influenza infection.

A potential challenge with i.n. delivery of targeted vaccines is the mucosal barrier which may block uptake of recombinant proteins. The mucus in the lung contains proteases that can degrade the vaccine molecules, and there are also mononuclear phagocytes present in the lung that may unspecifically phagocyte the vaccine. Nevertheless, we still observed specific staining of cDC1 with Xcl1-mCherry indicating that sufficient quantities of the vaccine reach the intended DC population. Whether the targeted Xcl1-mCherry molecules were directly taken up by the cDC1, for example through snorkeling mechanisms, or are transported across the epithelium by other means remains to be determined.

Our experiments indicate that targeting cDC1 by fusion to Xcl1 enhances CD4 and CD8 T cell responses in
both spleen and lung 1 week after immunization. These results are in accordance with our previous studies where dermal delivery of Xcl1 fusion vaccines, given as protein or DNA, enhance T cell responses.\textsuperscript{22,25} Interestingly, a recent study by Wakim and colleagues observed enhanced induction of tissue-resident memory T cells (T\textsubscript{RM}) in lung after i.n. immunization with OVA conjugated to anti-DEC205, which predominantly target cDC1.\textsuperscript{36} While longevity was not evaluated in this study, it is possible that targeting cDC1 can be utilized to enhance the induction of long-lived influenza-specific T cell responses in the lung. In this study, we focused on the influenza antigen haemagglutinin, which can induce both protective T cell and antibody responses. However, as HA vary

\textbf{FIGURE 4} Xcl1-HA protein vaccine confers protection in mice against a lethal dose of influenza PR8 virus. BALB/c mice were vaccinated intranasally with a single dose of 3 µg Xcl1-HA, αNIP-HA or HA with 50 µg poly(I:C) as earlier described and challenged 14 days later with 5 × LD50 influenza A/H1N1 PR8 virus. (A) Weight was monitored daily to follow disease progression, and mice were euthanized if they lost more than 20% of their starting weight. (B) Kaplan-Meier plot showing survival of mice presented in (A). (C) BALB/c mice were vaccinated intranasally with a single dose of 3 µg Xcl1-HA or αNIP-HA in the presence or absence of poly(I:C). After 2 weeks, serum samples were harvested and HA-specific IgG titres determined by ELISA. (D) Mice from (C) were challenged with a 5 × LD50 influenza A/H1N1 PR8 virus and monitored for weight loss over the course of 10 days. (E) Mice were immunized with Xcl1-HA or αNIP-HA as in (A), and challenged with H1N1pdm09 virus 2 weeks later. Weight was measured as a sign of disease progression. (A-B) Data from one of two representative experiments with n = 10-11 mice per group. (C-E) Data from one individual experiment with n = 5-6 mice in each group. (A and D) Two-way-ANOVA, (B) Mantel-Cox test. ** = P < .01, *** = P < .001
from season to season, thus necessitating annual influenza vaccination, immunization with more conserved antigens would be of interest. i.n. immunization with influenza antigens such as nucleoprotein or matrix could potentially provide cross-reactive T cells against numerous strains of influenza. 27,38 It should be noted that we did not observe heterologous protection against H1N1pdm09 virus after immunization with Xcl1-HA, although this may in part be related to the high pathogenicity of this strain and could potentially be improved upon by boosting with a second dose.

No difference was observed in the antibody response between Xcl1-HA and αNIP-HA immunized mice. There was a tendency for αNIP-HA immunized mice to induce stronger IgG1 responses, but the differences did not reach significance. We have previously seen that intradermal DNA vaccination with Xcl1-HA induces stronger IgG2a responses, 22,26,27,30 however, this was not seen in our i.n. immunization experiment. Most likely the addition poly(I:C), known to be a Th1 polarizing adjuvant, 40 masked the effect of using Xcl1 as a targeting unit. Immunization with Xcl1-HA significantly enhanced the induction of antibodies that could neutralize influenza virus, which is in contrast to previous studies where DNA vaccination with Xcl1-HA did not result in neutralizing responses. 26 Although the neutralizing titres were relatively low, the results support the idea that antibodies contribute more to protection after i.n. vaccination with adjuvant.

Interestingly, both Xcl1-HA and αNIP-HA induced significantly stronger antibody responses compared to monomeric HA. Indeed, one immunization with 3 µg HA did not induce detectable antibody responses. In contrast, immunization with a trimeric HA molecule readily induced antibody responses. Considering that HA is present as a monomer in the fusion vaccine molecule, 33 it appears that the bivalent format vaccine molecule is beneficial for antibody induction. These results are in accordance with previous observations made in our lab where DNA vaccination with a monomeric CCL3-fusion vaccine containing the tumour antigen M315 induced significantly lower antibody and T cell responses, and consequently poorer tumour protection, compared to a dimeric CCL3-M315 vaccine. 31 It should be noted that we did observe increased numbers of HA reactive T cells compared to PBS immunized mice, demonstrating that the monomeric HA was immunogenic.

Recent studies have demonstrated that CD8α+ and CD103+ DCs are the only known APC populations in mice that express the Xcr1 chemokine receptor. Interestingly, Xcr1 expression appears to be conserved on cross-presenting DCs in several different mammalian species, including humans, macaques and sheep. 13,14,41-43 Consequently, the immunization strategy presented in this study could easily be translated to humans or other mammals.

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CONFLICT OF INTEREST
The TTO office of Oslo University and Oslo University Hospital has filed several patents on bivalent fusion vaccine (Vaccibodies) on which BB is an inventor. BB is head of the scientific panel of the Vaccibody AS Company and holds shares in the company. The authors declare that there are no additional competing interests.

AUTHOR CONTRIBUTIONS
AL, AG, DT, SB and MB generated reagents and performed experiments. AL, BB and EF designed the experiments and conceptualized the study. AL, BB and EF wrote the manuscript, and all authors revised the manuscript. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT
Materials, data that support the findings and protocols will be made available to readers upon request to the corresponding author.

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