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REFERENCES

1. Dellon ES, Liacouras CA, Molina-Infante J, Furuta GT, Spergel JM, Zevit N, et al. Updated International Consensus Diagnostic Criteria for Eosinophilic Esophagitis: Proceedings of the AGREE Conference. *Gastroenterol*. 2018;155(4):1022-33 e10.
2. Travers J, Rochman M, Caldwell JM, Besse JA, Miracle CE, Rothenberg ME. IL-33 is induced in undifferentiated, non-dividing esophageal epithelial cells in eosinophilic esophagitis. *Sci Rep*. 2017;7(1):17563.
3. Judd LM, Heine RG, Menheniott TR, et al. Elevated IL-33 expression is associated with pediatric eosinophilic esophagitis, and exogenous IL-33 promotes eosinophilic esophagitis development in mice. *Am J Physiol Gastrointest Liver Physiol*. 2016;310(1):G13-25.
4. Hirano I, Dellon ES, Hamilton JD, et al. Efficacy of Dupilumab in a Phase 2 Randomized Trial of Adults With Active Eosinophilic Esophagitis. *Gastroenterology*. 2020;158(1):111-22 e10.
5. Doyle AD, Masuda MY, Kita H, Wright BL. Eosinophils in Eosinophilic Esophagitis: The Road to Fibrostenosis is Paved With Good Intentions. *Front Immunol*. 2020;11:603295.
6. Straumann A, Kristl J, Conus S, et al. Cytokine expression in healthy and inflamed mucosa: probing the role of eosinophils in the digestive tract. *Inflamm Bowel Dis*. 2005;11(8):720-726.

SUPPORTING INFORMATION

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Allergic eye disease: Blocking LTB4/C5 in vivo suppressed disease and Th2 & Th9 cells

To the Editor,

Vernal keratoconjunctivitis (VKC) affects children and can impair vision if the cornea becomes involved. Immunosuppressives (steroids and cyclosporin A) are required but can have side effects, and novel immunotherapeutic approaches are needed.[1] The aim of this study was to investigate the contributions of LTB4 and complement C5 in a model of allergic eye disease (experimental allergy conjunctivitis, EAC).[2] Previous studies have demonstrated that nomacopan, a bifunctional recombinant biologic derived from blood-feeding ticks, has anti-inflammatory properties by capturing LTB4 and preventing it from interacting with its two known G protein-coupled cell surface receptors (GPCR BLT1 and BLT2). Simultaneously, nomacopan inhibited C5 end terminal complement activation, thereby preventing formation of C5b-9 and C5a.[3] These two pathways have evolutionary connections as phylogenetic analysis showed that tick saliva started as an LTB4 inhibitor, and subsequently acquired the ability to inhibit C5. It has been shown in a mouse model of inflammatory arthritis that C5a production resulted in the release of LTB4 to promote further neutrophil migration to the interstitium.[4]

EAC is a model of allergic eye disease mainly driven by effector Th2 cells and mast cells (MC). During EAC, conjunctival inflammation can be detected after 5 days, scored non-invasively[2] (Figure S1A–C), with elevated levels of conjunctival IL-9-expressing $CD4^+$T cells and MC detected in tissues and cells expanded from conjunctival explants (Figure S1D–J). Significantly increased levels of $tryptase^+$ conjunctival MC were observed, co-expressing intracellular IL-9 in the sub-epithelial area within the fornix of conjunctival EAC tissues (Figure S1G).

In this study, we investigated the effects of topically administered nomacopan in EAC and observed a significant suppression of disease in treated mice, and a decrease in IL-9-expressing $CD4^+$T cells (Figure 1A–C; Figure S2A). Recent studies have demonstrated that IL-9 can be produced by Th9 and Th2 cells.[5,6] To determine which $CD4^+$T cell subset was producing IL-9, transcription factor expression was investigated. IL-9-producing Th2 cells express GATA3, but not PU-1, whereas IL-9-producing Th9 cells express PU.1, but not GATA3. Within the infiltrating $CD4^+$T cells in EAC, Th9 cells ($IL\text{-}9^+PU.1^+$) and IL-9-expressing Th2 cells

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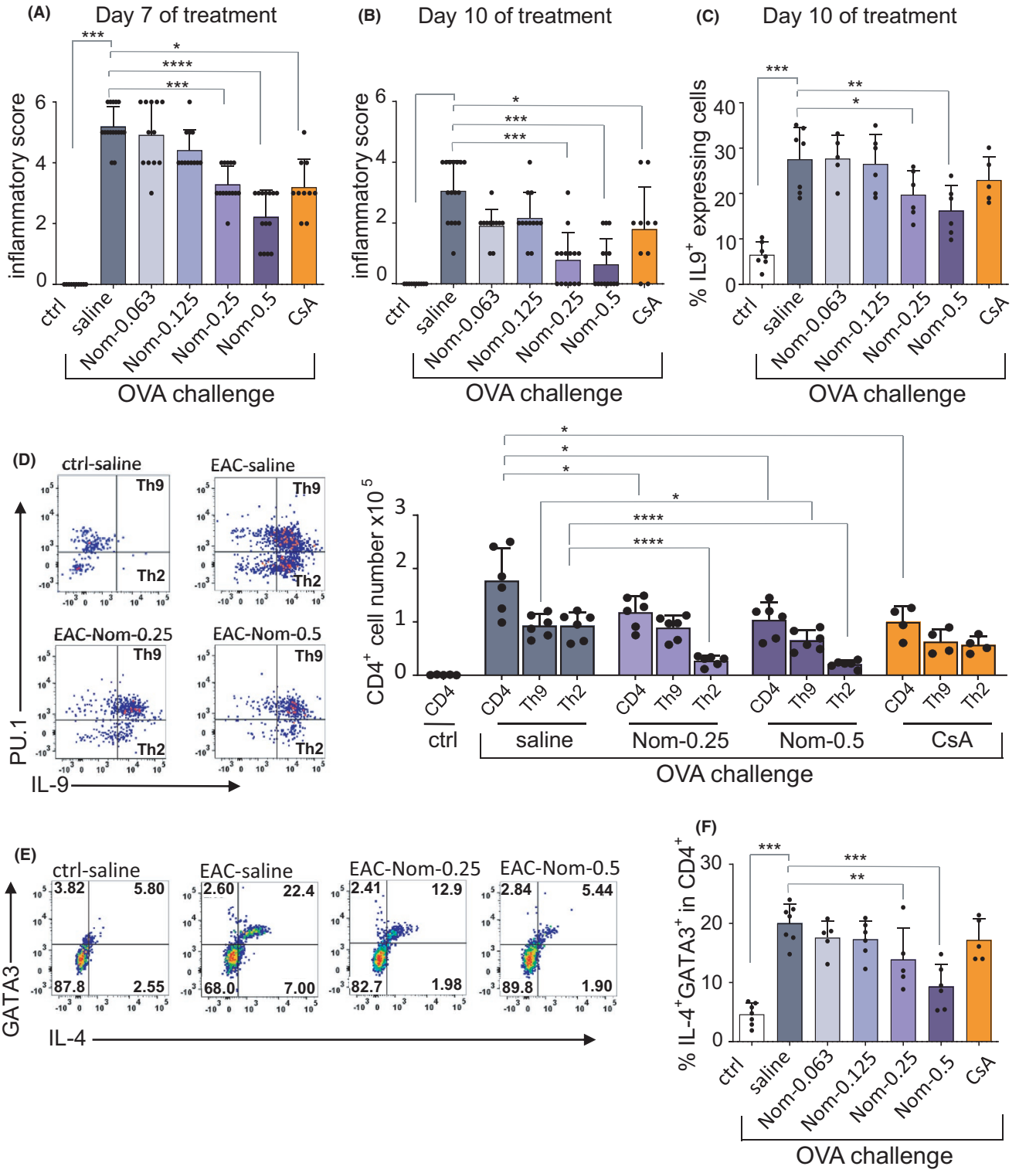

FIGURE 1 Topical nomacopan attenuated conjunctivitis in EAC and decreased IL-9 in $CD4^+$T cells. (A, B) Clinical scores of eyes were compared with saline-treated controls. (C–F) Conjunctival explant cells stained for T-cell markers. Flow cytometry plots represent staining for: (C) IL-9, (D) IL-9 and PU.1 (Th9 cells) and a summary graph of percentages for $IL\text{-}9^+PU.1^+$ (Th9) or $IL\text{-}9^+PU.1^-$ (Th2) cells. (E, F) Representative plots were gated for Th2 cells ($IL\text{-}4^+GATA3^+PU.1^-$) in treatment groups and a summary graph. Four independent experiments, ANOVA and unpaired *t*-tests; mean ± SD ($n = 6$–8 mice per group). $^*p < .05$, $^{**}p < .01$. Nom = nomacopan, CsA = Cyclosporine A

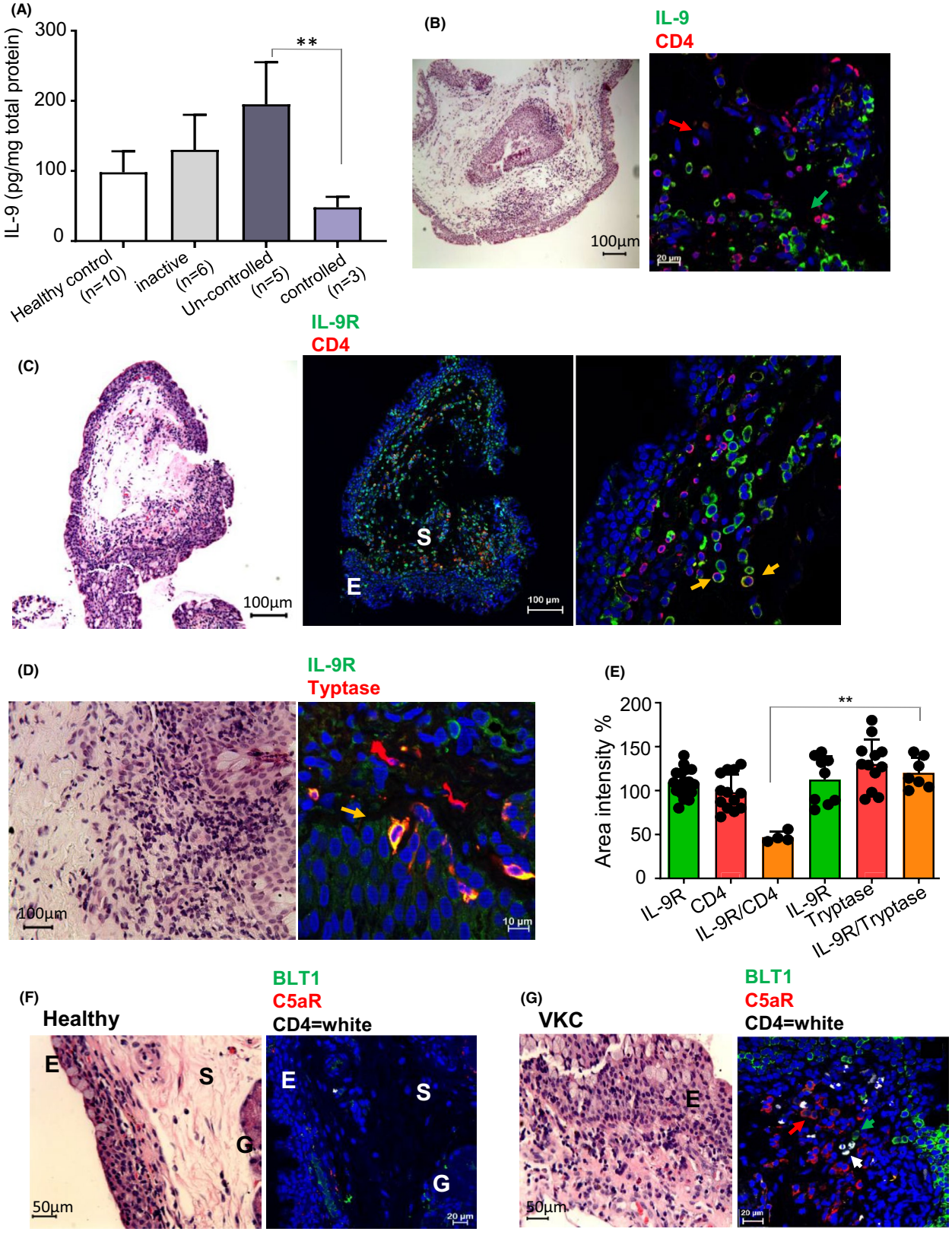

FIGURE 2 (A) IL-9 levels in tear fluids, demonstrating an increase in clinically uncontrolled (i.e. active) VKC as compared with those with clinically controlled disease; (B) Histology/immunofluorescence staining of VKC conjunctival sections for CD4 and IL-9; (C) CD4 and IL-9R; (D) Tryptase and IL-9R. (E) Scatterplots based on immunoreactivity and intensity for IL-9R, tryptase or IL-9R and CD4 as percentages of the immunostained areas. Representative summary graph of one of 7 biopsies. Values were added from 3 fields. (F, G) Representative staining for C5aR1, BLT1, CD4 on healthy, 1 of 3 (F) and VKC 1 of 7 (G) conjunctival tissues. Mean ± SD, ANOVA, $^{**}p < .01$,$^{**}p < .001$. E = epithelial layer; S = stroma: G = gland (Lacrimal)

(GATA3$^+$IL-4$^+$IL-9$^+$) cells were increased in OVA-challenged mice compared with controls (Figure S1H–J).

During early stages of EAC (Days 1–6), no effect by nomacopan was observed due to low inflammatory scores overall. However, at days 7 and 10, a significant decrease in IL-9$^+$CD4$^+$ T cells (Figure 1C–F) following nomacopan treatment was observed. The levels of Th2 (IL-4$^+$ PU.1$^-$ GATA3$^+$) cells and IL-9-expressing Th2 cells were also significantly reduced (Figure S2B–D). Interestingly, although there was an overall reduction in IL-9 expression levels, there were significant changes in Th9 (IL-9$^+$PU.1$^+$IL-4$^-$) levels only at the higher concentration of nomacopan (Figure 1D).

This *in vivo* model shares many features with VKC in man since there is a predominant CD4$^+$T cell infiltration of the conjunctival tissues and evidence of fibrosis.[2] Hence, we used VKC tissue specimens and tear fluids to compare with EAC tissues. Tears collected from VKC patients during active disease showed a significantly higher level of IL-9 (clinically uncontrolled) as compared with inactive VKC (controlled; Figure 2A). VKC conjunctival tissue sections were examined for expression of CD4, IL-9, IL-9 receptor (IL-9R), and tryptase (Figure 2B–E). IL-9-expressing CD4$^+$T cells and MC were both significantly up-regulated in all active VKC specimens (n = 7) as compared with controls. We observed CD4$^+$T cells co-localized with IL-9 in the sub-epithelial and stromal areas of VKC sections (Figure 2B) and IL-9R expression (Figure 2C). Infiltrating MC (Tryptase$^+$) and other cell types also expressed IL-9R (Figure 2D) within stromal areas, with the frequency of IL-9R-expressing MC significantly higher than IL-9R-expressing CD4$^+$T cells (Figure 2E).

To further investigate whether the receptors for those ligands targeted by nomacopan were expressed during EAC, conjunctival tissue expression and localization of C5aR and BLT1 (LTB4 receptors) were investigated (Figure S2E) as well as in VKC and healthy tissue controls (Figure 2F,G). Relative expression of BLT1 and C5aR was analysed by counting positively stained cells in at least 5 non-overlapping areas (ImageJ). Interestingly, increases in infiltrating immune cells within the stromal areas correlated with increased expression of BLT1 (means ± SD; 34.98 ± 15) and C5aR (56.67 ± 12) in EAC, and comparable levels were also observed in VKC: BLT1 (40.48 ± 10); C5aR (44.90 ± 20). Very few cells co-expressed both receptors while only rarely were any BLT1$^+$ or C5aR1$^+$ cells detected in healthy mouse and human tissues, suggesting that these receptors are exclusively expressed during disease, potentially by infiltrating CD4$^+$T cells and MC.

We conclude that IL-9 was up-regulated during VKC and EAC. Nomacopan significantly suppressed EAC severity, accompanied by a decrease in IL-9-producing Th2 cells, Th2 cells and to a lesser extent, Th9 cells, suggesting a key pro-inflammatory role for IL-9-secreting CD4$^+$T cells in allergic eye disease.

Our findings support nomacopan as a potential treatment for allergic eye disease due to its ability to down-regulate LTB4/C5 pathways in EAC.

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CONFLICT OF INTEREST

No authors have any Conflicts of Interest with the exception of Drs. Nunn and Weston-Davies, who are both employees of Akari Therapeutics Inc.

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REFERENCES

1. Leonardi A, Lazzarini D, Motterle L, et al. Vernal keratoconjunctivitis-like disease in adults. *Am J Ophthalmol*. 2013;155(5):796-803. doi:10.1016/j.ajo.2012.11.018
2. Reyes NJ, Mathew R, Saban DR. Induction and characterization of the allergic eye disease mouse model. *Methods Mol Biol*. 2018;1799:49-57. doi:10.1007/978-1-4939-7896-0_5
3. Roversi P, Ryffel B, Togbe D, et al. Bifunctional lipocalin ameliorates murine immune complex-induced acute lung injury. *J Biol Chem*. 2013;288(26):18789-18802. doi:10.1074/jbc.M112.420331
4. Sidik CD, Miyabe Y, Sezin T, Luster AD. The critical role of C5a as an initiator of neutrophil-mediated autoimmune inflammation of the joint and skin. *Semin Immunol*. 2018;37:21-29. doi:10.1016/j.smim.2018.03.002
5. Micosse C, von Meyenn L, Steck O, et al. Human "TH9" cells are a subpopulation of PPAR-gamma(+) TH2 cells. *Sci Immunol*. 2019;4(31):1-13. 10.1126/sciimmunol.aat5943
6. Chang HC, Sehra S, Goswami R, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol*. 2010;11(6):527-534. https://doi.org/10.1038/ni.1867

SUPPORTING INFORMATION

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Virus-like particles displaying recombinant Der p 1 zymogen to optimize IgG blocking antibody response

To the Editor,

Successful allergen-specific immunotherapy (AIT) was shown to be correlated with the levels of blocking IgG antibodies. As repetitive arrays of antigens in virus-like particles (VLPs) promote potent neutralizing antibody responses, we multimerized the major house dust mite (HDM) allergen Der p 1 on the surface of bacteriophage AP205 VLPs using the Split-protein (SpyCatcher-SC/SpyTag-ST) technology.[1] As recombinant folded Der p 1 is only obtained through the production of Der p 1 zymogen (ProDer p 1, pDp1),[2] pDp1C34N52Q-L-ST (pDp1C) and ST-ExtL-pDp1C34AN52Q (pDp1N) (Der p 1 numbering) were produced in *P. pastoris* (Figure 1A,B). Mutations of active site cysteine residue (C34A) and mature Der p 1 N-glycosylation site (N52Q) prevented hypermannosylation of pDp1[2] and any proteolytic degradation of SC/ST due to some levels of cysteine protease activity in WT pDp1-ST preparations (Figure S1). ST was fused to N- or C-terminus of pDp1 to evaluate the impact of allergen orientation on the immunogenicity of chimeric VLPs. pDp1N and pDp1C were successfully conjugated to SC-VLP under acidic conditions: 65 kDa protein bands matching with the theoretical size of SC-VLP subunit (27 kDa): pDp1 (37 kDa) conjugates were detected by SDS-PAGE (Figure 1B). Densitometric analysis evidenced that each VLP-pDp1N and VLP-pDp1C particle displays 150 and 180 pDp1 copies, respectively. Dynamic light scattering (DLS) analysis showed that VLP-pDp1N and VLP-pDp1C have a hydrodynamic diameter of 118 and 112 nm, respectively, and with 26%–27% polydispersity (%Pd) (Figure S2A).

Competitive ELISA IgE assays showed that, in solution, both VLP-pDp1 particles had a much lower IgE reactivity in comparison with natural Der p 1 (nDer p 1) or monomeric SpyTagged-pDp1 ($p < 0.05$) (Figure 1C). However, indirect ELISA evidenced that VLP-pDp1 and nDer p 1, coated at equimolar concentrations of Der p 1, similarly bind Der p 1-specific human IgE (Figure S3). RBL-SX38 mediator release assays evidenced that the basophil degranulation capacity of both VLP-pDp1 is almost completely abolished with the exception of partial basophil activation at a 1μg/ml concentration for some tested sera ($p < 0.05$) (Figure 1D).

In Balb/c mice (Figure 2A), intramuscular immunizations with unadjuvanted VLP-pDp1N and VLP-pDp1C triggered potent pDp1-specific IgG1/IgG2a responses (Figure 2B). The allergen multimerization dramatically increased pDp1 immunogenicity as judged by the much lower antibody levels induced with equimolar amounts of monomeric pDp1N or pDp1C mixed with untagged VLPs ($p < 0.05$). Importantly, specific IgG1/IgG2a antibodies were similarly capable to recognize nDer p 1 ($p > 0.05$) (Figure 2C). The low-specific IgG1/IgG2a ratio together with the absence of detectable pDp1/nDer p 1-specific IgE in sera from VLP-pDp1-immunized mice (data not shown) suggested that both unadjuvanted particles promote Th1-biased humoral responses. The multimeric display of pDp1 in repetitive arrays elicited as well Th1-biased cellular responses characterized by large IFNγ production from pDp1-restimulated spleen cells isolated from mice immunized with both VLP-pDp1 ($p > 0.05$) (Figure 2D). The pro-Th1 adjuvant capacity of bacterial RNA packaged in the chimeric VLPs mediated the Th1 polarization[3] (Figure S2B).

Both VLP-pDp1-induced IgG antibodies similarly inhibited the binding of human-specific IgE to coated nDer p by around 60% ($p > 0.05$) (Figure 2E). In RBL-SX38 assays, nDer p 1-stimulated β-hexosaminidase release was inhibited by 80% and 65% when the allergen was preincubated in a 1/100 dilution of mouse sera from VLP-pDp1N- or VLP-pDp1Cimmunized mice, respectively ($p < 0.05$)