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The Purinergic G Protein-Coupled Receptor 6 Inhibits Effector T Cell Activation in Allergic Pulmonary Inflammation

Giorgio Giannattasio,^{*,†,‡} Shin Ohta,^{*,†,‡} Joshua R. Boyce,[†] Wei Xing,^{*,†} Barbara Balestrieri,^{*,†} and Joshua A. Boyce^{*,†,§}

We show that the P2Y₆ receptor, a purinergic G protein-coupled receptor with a high affinity for the nucleotide uridine diphosphate, is an important endogenous inhibitor of T cell function in allergic pulmonary inflammation. Mice conditionally deficient in P2Y₆ receptors [*p2ry6* (flox/flox);cre/+ mice] exhibited severe airway and tissue pathology relative to P2Y₆-sufficient [*p2ry6* (flox/flox)] littermates (+/+ mice) when treated intranasally with an extract of the dust mite *Dermatophagoides farinae* (Df). P2Y₆ receptors were inducibly expressed by lung, lymph node, and splenic CD4⁺ and CD8⁺ T cells of Df-treated +/+ mice. Df-restimulated P2Y₆-deficient lymph node cells produced higher levels of Th1 and Th2 cytokines, and polyclonally stimulated P2Y₆-deficient CD4⁺ T cells proliferated faster than comparably stimulated P2Y₆-sufficient cells. The absence of P2Y₆ receptors on CD4⁺ cells, but not APCs, was sufficient to amplify cytokine generation. Thus, P2Y₆ receptors protect the lung against exuberant allergen-induced pulmonary inflammation by inhibiting the activation of effector T cells. *The Journal of Immunology*, 2011, 187: 000–000.

Nucleotides, the structural subunits of the nucleic acids, are also important extracellular signaling molecules (1, 2). They are stored in cytosolic and secretory compartments of cells and are released in response to cell injury, hypoxia, shear stress, and tissue injury (3–5). Nucleotides are also released by platelets, endothelial cells, mast cells (MCs), macrophages, and T cells in response to physiologic activation (6–9). Consequently, extracellular nucleotides accumulate at sites of vascular injury, hypoxia, thrombosis, inflammation, and immune cell activation. Both adenine-containing (ATP and ADP) and uracil-containing (UTP and UDP) nucleotides have extracellular functions mediated by cognate cell surface receptors. These receptors fall into two classes: purinergic (P2)X receptors, which are ligand-gated ion channels that mediate calcium and potassium fluxes in response to ATP, and P2Y receptors, which are G protein-coupled receptors (10–12). Although adenine nucleotides and their re-

ceptors play established roles in platelet aggregation (13), pain perception (14), and cellular responses to hypoxic injury (15), the functions of many P2Y receptors in vivo remain unknown.

P2Y₆ receptors are the only known high-affinity receptors selective for UDP. They are expressed on both hematopoietic (macrophages, dendritic cells, MCs, and T cells) and nonhematopoietic (vascular smooth muscle, epithelium, and endothelium) cell types (16–25). Most functions attributed to P2Y₆ receptors in vitro suggest a role in modulating cellular responses to inflammation through autocrine or paracrine actions of uracil nucleotides. Endogenous uracil nucleotides amplify airway epithelial chemokine production in response to neutrophil-derived peptides through P2Y₆ receptors in vitro (26). P2Y₆ receptors are strongly expressed by colonic epithelial cells in biopsies from patients with inflammatory bowel disease and mediate production of IL-8 by colonic epithelial cell lines in vitro (19). P2Y₆ receptors amplify chemokine generation by a LPS-stimulated monocytic cell line (27), amplify endothelial cell activation in response to LPS (28), and enhance the release of macrophage inflammatory protein-1 β by human MCs stimulated with leukotriene D₄ (20). Human T cells express P2Y₆ receptors after activation in vitro and at sites of inflammation in vivo (23), and pharmacologic blockade of P2Y₆ receptors inhibits proliferation, CD25 expression, and generation of cytokines by mouse T cells in response to polyclonal and Ag-specific stimulation (9, 25). Although these in vitro studies suggest physiologic functions for P2Y₆ receptors in immune responses and inflammation, not all such functions have been demonstrated in vivo.

Given its distribution on cells of both the innate and adaptive immune systems, we suspected that P2Y₆ receptors might play a role in the pathogenesis of allergic pulmonary inflammation. In this study, we demonstrate that P2Y₆ receptors are endogenous suppressors of T cell-driven pulmonary pathology induced by exposure to the allergens of the house dust mite *Dermatophagoides farinae*, which are potent sensitizers in asthma. Newly created C57BL/6 (B6) mice bearing a conditional deletion of the P2Y₆ receptor [*p2ry6* (flox/flox);cre/+ mice] displayed sharply

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Abbreviations used in this article: B6, C57BL/6; BAC, bacterial artificial chromosome; BAL, bronchoalveolar lavage; BVB, bronchovascular bundle; Df, extract from *Dermatophagoides farinae*; MC, mast cell; P2, purinergic; PAS, periodic acid-Schiff; PLN, parabrachial lymph node; P2Y, purinergic G protein-coupled receptor; qPCR, quantitative PCR.

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increased pulmonary inflammation, lymph node hyperplasia, and allergen-induced Th1 and Th2 recall responses compared with P2Y₆-sufficient littermate controls [*p2ry6* (flox/flox);^{+/+}, (^{+/+}) mice] when exposed intranasally to an extract of *D. farinae* (*Df*). P2Y₆ receptors were constitutively expressed at low levels by alveolar and lung interstitial macrophages but absent on splenic and parabrachial lymph node (PLN) T cells from naive mice. However, P2Y₆ receptor expression was induced on both CD4⁺ and CD8⁺ T cells from PLNs of ^{+/+} mice exposed to *Df*. The absence of P2Y₆ receptors from CD4⁺ T cells of *Df*-challenged mice, but not from lung-derived macrophages used as APCs, was sufficient to increase Th1 and Th2 cytokine generation in an Ag restimulation assay. Thus, the inducible expression of P2Y₆ receptors by T cells permits endogenous UDP to modify cellular activation and cytokine production, dampening the inflammatory response to the clinically relevant allergens of house dust mites.

Materials and Methods

Mice

A 12.7-kb region of the *p2ry6* gene was subcloned from a B6 bacterial artificial chromosome (BAC) clone using homologous recombination. A single loxP site was inserted at the 5' side of exon 3 and a Neo cassette flanked by FRT sites and loxP sites was inserted at the 3' of exon 3. The region of the targeting vector was designed such that the long (LA) and the short (SA) homology arms extended 9.6 kb at the 5' side and 2.1 kb at the 3' of the cassette, respectively. The structure of the targeting vector was confirmed by restriction analysis after each modification step and by sequencing using primers designed to read from the selection cassette into the 3' of the LA (N7) and the 5' end of the SA (N1) and primers annealing to the vector sequence (P6 and T7) and reading into the 5' and 3' ends of the BAC subclone. The BAC was subcloned into a 2.4-kb subclone vector containing an ampicillin cassette for retrotransformation of the construct prior to electroporation. The resultant targeting vector was linearized and electroporated into 129/Sv mouse embryonic stem cells, and cells were positively selected with G418. Six verified embryonic stem clones were microinjected into blastocysts from B6 mice, and chimeric mice were obtained and crossed with B6 mice to produce male and female *p2ry6* (flox/+) mice. To generate *p2ry6* (flox/flox) and *p2ry6* (flox/flox);*cre*/+ mice on a B6 × 129 mixed background, *p2ry6* (flox/+) mice were intracrossed to produce *p2ry6* (flox/flox) mice. *P2ry6* (flox/flox) mice were then mated with B6 mice homozygous for the Cre recombinase (*cre/cre*, B6 rosa26CreER(T2); Taconic-Artemis, Cologne, Germany) to obtain *p2ry6* (flox/+) *cre*/+ mice that were then mated with *p2ry6* (flox/flox) mice to produce the experimental mice *p2ry6* (flox/flox) (^{+/+}) mice and *p2ry6* (flox/flox);*cre*/+ (Fig. 1B). Once *p2ry6* (flox/flox) and *p2ry6* (flox/flox);*cre*/+ were obtained, they were also used as breeders for the maintenance of the colony (Fig. 1). The Cre recombinase was induced in 5- to 11-wk-old *p2ry6* (flox/flox);*cre*/+ mice by administering tamoxifen (1 mg; Sigma-Aldrich, St. Louis, Mo), dissolved in a mixture of ethanol/sunflower seed oil (1:4.5, v/v), by i.p. injections on 5 consecutive days (29). Cohorts of sex- and age-matched *p2ry6* (flox/flox) mice were also administered tamoxifen simultaneously to ensure equal treatment of the two strains. Tamoxifen-mediated induction of Cre recombinase in *p2ry6* (flox/flox);*cre*/+ mice resulted in the disruption of the *p2ry6* gene and a loss of protein expression (Fig. 2). Mice were housed under pathogen-free conditions and maintained on a 12-h light/dark cycle for the entire duration of the studies. All the studies described in this paper were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA).

Induction of pulmonary inflammation

Ten days after the administration of tamoxifen, pulmonary inflammation was induced in age (7–13 wk)- and sex-matched ^{+/+} and *p2ry6* (flox/flox);*cre*/+ mice by intranasal instillation of 3 µg *Df* (Greer Laboratories, Lenoir, NC) in 20 µl 0.9% NaCl (containing <0.005 EU/ml endotoxin; Sigma-Aldrich) twice a week for 3 wk, as described previously (30, 31). In selected experiments, the amount of *Df* was lowered to 1 µg/dose. Control groups of ^{+/+} and *p2ry6* (flox/flox);*cre*/+ mice received 0.9% NaCl alone. The same protocol was used for the experiments with the *cre*/+ mice. Twenty-four hours after the last treatment, mice were euthanized, and blood was collected by cardiac puncture and centrifuged to obtain serum. Bronchoalveolar lavage (BAL) was performed with three aliquots of 0.7 ml ice-cold PBS containing 10% FBS and 0.5 mM EDTA. BAL fluid cells

were cytocentrifuged onto slides, stained with Diff-Quick (Fisher Diagnostic, Middletown, VA), and differentially counted as mononuclear cells (monocytes/macrophages and lymphocytes), neutrophils, or eosinophils, according to the standard hemocytological criteria.

Histological evaluation of pulmonary inflammation

Left lungs were harvested at the time of euthanasia, fixed, and embedded in glycolmethacrylate or in paraffin (32). Two-micrometer-thick glycolmethacrylate sections were stained with H&E or by the periodic acid-Schiff (PAS) reaction to depict mucus-secreting epithelial cells (goblet cells). The extent of cellular infiltration in the bronchovascular bundles (BVBs) and the number of PAS-positive goblet cells were evaluated from each animal in the experimental groups by a pathologist blind to mouse strain and procedure.

Real-time quantitative PCR of mRNA transcripts in the lung and PLNs

Right lungs were collected at time of euthanasia and snap-frozen. Freshly isolated PLN cells were pooled from five to seven mice per group. Total RNA was isolated from tissue homogenates and from PLN cells with Tri-Reagent (Sigma-Aldrich), reverse transcribed into cDNA (RT² First Strand kit; SABiosciences, Frederick, MD), and assayed by real-time quantitative PCR (qPCR) for mouse P2Y₆, IL-5, IL-13, the mucus-associated proteins Muc5ac and Clca3/Gob-5, the transcription factors T-bet, GATA-3, Foxp3, and ROR-γT, the chemokines CCL2, CCL11, and CXCL2, and GAPDH on an Mx3005P thermal cycler (Stratagene) with the use of SYBRGreen/ROX master mix (SABiosciences). The ratio of each mRNA relative to the GAPDH mRNA was calculated with the ΔΔCt method. The primers used for mouse P2Y₆ (16), IL-5, IL-13, Muc5ac, Clca3/Gob-5, and GAPDH were reported previously (31). For the transcription factors, the fold induction was calculated based on the ratio of corrected expression in the samples from *Df*-treated mice and from saline-treated controls.

Immunohistochemical analysis of P2Y₆ in lungs, PLNs and spleen

Sections of paraffin-embedded lungs, PLNs and spleens were deparaffinized and rehydrated. Ag retrieval was performed with Target Retrieval Solution (DakoCytomation, Glostrup, Denmark) at 97°C for 30 min. After blocking with 10% chicken serum (Santa Cruz Biotechnology, Santa Cruz, CA), sections were incubated (1 h, 37°C) with 43 µg/ml of a rabbit anti-P2Y₆ Ab (20) and then with the peroxidase-conjugated rabbit ABC staining system (Santa Cruz Biotechnology), according to the manufacturer's instructions. Slides were analyzed with a Leica DM LB2 microscope (Leica Microsystems, Wetzlar, Germany), and pictures were captured with a Nikon digital camera DXM 1200 with Nikon ACT-1 (version 2.70) image acquisition software.

Flow cytometry analysis of PLN cells

Pooled PLNs from NaCl- and *Df*-treated ^{+/+} and *p2ry6* (flox/flox);*cre*/+ mice were homogenized and red cells were lysed. The cells obtained were fixed, permeabilized by incubation in FACS buffer (PBS buffer containing 0.5% BSA [Sigma-Aldrich]) plus 0.1% saponin (from *Quillaja saponaria*; Sigma-Aldrich) and blocked in FACS buffer containing 10% normal mouse serum and 1% anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences, San Jose, CA). Cells were washed and incubated (45 min, 4°C) with the following Abs: allophycocyanin-CD4 (clone RM4-5), allophycocyanin-CD8b (H35-17.2), PE-Cy7-CD11c (N418), PerCP-Cy5.5-B220 (RA3-6B2) (eBioscience), and Alexa Fluor 488-labeled (Zenon Alexa Fluor 488 rabbit IgG labeling kit, Invitrogen) rabbit anti-P2Y₆ [10 µg/ml, directed against a conserved peptide within the second intracellular loop of the human P2Y₆ receptor (20). The corresponding isotypes (eBioscience) or Alexa Fluor 488-labeled normal rabbit IgG (Jackson ImmunoResearch Laboratories) were used as controls. Annexin V staining was performed as previously described (33) on the CD4⁺ gate. The acquisition was performed on a FACSCanto flow cytometer with FACSDiva software (BD Biosciences), and data were analyzed with FlowJo (Tree Star, Ashland, OR).

In vitro restimulation of lymph node cells with *Df*

PLNs were collected from the upper-right chest of each mouse and homogenized in complete medium (RPMI 1640 medium, 10% FBS, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME [Sigma-Aldrich]), plus 25 mM HEPES, and 1 mM sodium pyruvate. The red cells were lysed, and 4 × 10⁶ nucleated cells were incubated for 72 h with medium alone or containing 20 µg/ml

Df (30). The concentrations of IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN- γ released in the supernatants were measured by ELISA (eBioscience, San Diego, CA). The amount of cytokine per 10^6 PLN cells and the total number of PLN cells were used to calculate the total amount of cytokine generated per lymph node.

For the ELISPOT analysis, 2.5×10^5 PLN-nucleated cells were incubated in medium containing *Df* (20 μ g/ml) for 72 h on 96-well cell culture plates with polyvinylidene difluoride filter membranes (Millipore, Billerica, MA) and precoated with Abs against IL-4, IL-5, IL-13, IL-17A, and IFN- γ (eBioscience). The spots were detected with an ELISPOT kit (eBioscience), according to the manufacturer's instructions, and counted with a CTL Immunospot Analyzer (Cellular Technology, Shaker Heights, OH). For each cytokine the total number of producing cells was calculated by multiplying the frequency of producing cells by the total number of PLN cells.

PLN CD4⁺ T cell proliferation

PLN cells from NaCl- or *Df*-treated *+/+* and *p2ry6* (flox/flox);cre/+ mice were obtained, incubated (10 min, 37°C) in PBS with 1 μ M CFDA-SE (eBioscience), and extensively washed in culture medium. A total of 4×10^6 CFDA-SE-labeled cells were then added to 24-well plates precoated with an anti-CD3 Ab (1 μ g/ml overnight) and cultured in the presence of soluble anti-CD28 Ab (1 μ g/ml) for 72 h. At the end of the incubation, cells were harvested, washed in FACS buffer, blocked, and incubated (45 min, 4°C) with allophycocyanin-CD4 Ab (RM4-5; eBioscience). Data were acquired on a FACSanto flow cytometer with FACS Diva software. The CFDA-SE profile was evaluated on the CD4⁺ gate and analyzed with FlowJo. The cells falling into the lowest CFSE peak were counted as "proliferating."

Isolation of lung macrophages and T cells for confocal microscopy

Lung macrophages were obtained using a modification of previous protocols (34, 35). After airway cells and alveolar macrophages were removed by extensive BAL with PBS containing EDTA, lungs were homogenized through a 70- μ m mesh in ice-cold complete medium, washed, and incubated (30 min, 37°C) with 428 U/ml collagenase IV (Worthington, Lakewood, NJ) and 20 μ g/ml DNase I (Roche, Mannheim, Germany). The obtained cell suspension was washed and layered onto a Percoll gradient (60 and 40%). After centrifugation, the cells at each interface were collected, washed, and centrifuged again on a second density gradient. At the end of the procedure, the obtained cells, representing an enriched (>85%) population of interstitial macrophages, were stained with Diff-Quick (Fisher Diagnostic) or analyzed by flow cytometry with the following Abs: allophycocyanin-CD68 (clone FA-11; AbD Serotec), FITC-MHC-II (M5/114.15.2), PE-Cy7-CD11c, (N418), PE-Cy7-CD3e (145-2C11), FITC-CD4 (RM4-5), PE-Cy7-CD8a (53-6.7), and PerCP-Cy5.5-B220 (RA3-6B2) (eBioscience). CD4⁺ cells were purified from pooled lungs of five mice per group using a FACS Aria II high-speed cell sorter (Dana-Farber Cancer Institute Flow Cytometry Core). For confocal microscopy analysis, the macrophages were further enriched by adherence (overnight, 37°C) on coverslips. After nonadherent cells were removed by washing, adherent macrophages were fixed with 2% paraformaldehyde (Sigma-Aldrich) and permeabilized with PBS containing 0.1% saponin (36). Cells were stained with goat polyclonal anti-P2Y₆ Ab or goat IgG as negative control (10 μ g/ml; Santa Cruz Biotechnology), and counterstained with FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Z-stack images were acquired with a Nikon C1 plus laser scanner confocal system combined with an Eclipse TE2000-U inverted microscope with a $\times 60$ oil PlanApo NA 1.4 objective lens (36).

Cocultures of lung macrophages and splenic CD4⁺ T cells

To purify CD4⁺ T cells, spleens of NaCl- or *Df*-treated *+/+* and *p2ry6* (flox/flox);cre/+ mice were homogenized, and nucleated cells were blocked and incubated (45 min, 4°C) with PE-Cy7-CD3e (clone 145-2C11) and FITC-CD4 (RM4-5) Abs (eBioscience). The double-positive CD3⁺CD4⁺ cells were sorted by flow cytometry (Dana-Farber Cancer Institute Flow Cytometry Facility, Boston, MA). Lung interstitial macrophages (1×10^5 /ml) and CD3⁺CD4⁺ cells (7.5×10^5 /ml) were cocultured in either medium alone or containing 20 μ g/ml *Df* for 72 h. At the end of the incubation, the concentrations of IL-5, IL-13, and IFN- γ released in the supernatants were measured by ELISA (eBioscience).

Statistics

Nonparametric Mann-Whitney *U* test and Kruskal-Wallis test with Dunn's posttest correction for multiple comparisons were used to compare two and

three or more groups, respectively. Analysis was performed with Prism software (GraphPad, La Jolla, CA). The *p* values <0.05 were considered significant.

Results

Deletion of P2Y₆ receptors increases pulmonary inflammation induced by *Df* allergen

The *p2ry6* allele was deleted by flanking the gene with P-lox sites (Fig. 1A). The *p2ry6* (flox/flox);cre/+ mice and their *+/+* littermate controls (Fig. 1B) were treated with tamoxifen for 5 consecutive d i.p. PCR analysis confirmed the genotype of the mice used in the experiments (Fig. 1C). Immunohistochemistry confirmed the absence of the P2Y₆ receptor protein in the lungs, PLN, and spleen of the *p2ry6* (flox/flox);cre/+ mice after tamoxifen treatment (Fig. 2). The initial experiments were performed with F₁-F₅ B6 $\times$ 129 mice and littermate controls. The results obtained in these experiments were similar to those obtained after backcrossing the mice for 10 generations to the B6 background (Fig. 3A).

To determine whether P2Y₆ receptors played a role in the development of allergic pulmonary inflammation, *DF* (3 μ g protein) or saline was administered intranasally, twice weekly for 3 consecutive wk (25) to cohorts of *+/+* and *p2ry6* (flox/flox);cre/+ mice after both genotypes were treated with tamoxifen. *Df* caused increased BAL fluid cell numbers, eosinophils, neutrophils, macrophages, and lymphocytes. However, the total number of cells in BAL fluid of the *Df*-treated *p2ry6* (flox/flox);cre/+ mice exceed that of the *+/+* controls by >2-fold (Fig. 3A). Compared with the *Df*-treated *+/+* controls, the *Df*-treated *p2ry6* (flox/flox);cre/+ mice showed ~5-fold higher numbers of BAL fluid eosinophils and neutrophils (Fig. 3A). Similar results were obtained after backcrossing the initial F₁ mice to the B6 background for 10 generations (Fig. 3B). Tamoxifen-treated Cre/+ mice without the fluxed *p2ry6* allele showed identical degrees of *Df*-induced inflammation, PLN cell counts, and PLN cytokine production to wild-type controls (Supplemental Fig. 1A–C, respectively), indicating that activation of Cre recombinase per se did not alter the phenotype.

Because the peribronchial inflammation caused by *Df* at 3 μ g was too severe to show substantial differences between the genotypes (data not shown), we reduced the dose of *Df* to 1 μ g in selected experiments to determine whether the deletion of P2Y₆ receptors altered pulmonary pathology at a lower dose of Ag. At this allergen dose, the BVBs of the *p2ry6* (flox/flox);cre/+ mice contained more extensive inflammatory infiltrates consisting of mononuclear cells along with eosinophils, neutrophils, lymphocytes, and plasma cells than did the *+/+* controls (Fig. 3C). In addition, the bronchial epithelium of the *p2ry6* (flox/flox);cre/+ mice demonstrated more goblet cell metaplasia than did the *+/+* controls (Fig. 3C). Higher levels of expression of IL-5 and IL-13 mRNA transcripts were present in the lungs of the *Df*-treated *p2ry6* (flox/flox);cre/+ mice than in the lungs of the *+/+* mice, with similar trends for the goblet cell-associated transcripts Muc5ac and Clca3/Gob-5 (Fig. 3D). There were no significant differences in the levels of transcripts encoding CCL2 (a monocyte chemoattractant), CCL11 (an eosinophil chemoattractant), or CXCL1 (a neutrophil chemoattractant) (Supplemental Fig. 2) between the lungs of the genotypes.

Cellular distribution of P2Y₆ receptors in the lungs and lymphoid tissues

Because the deletion of P2Y₆ receptors resulted in increased pulmonary inflammation in response to *Df*, we sought to determine the cellular distribution of P2Y₆ receptors in the lungs and

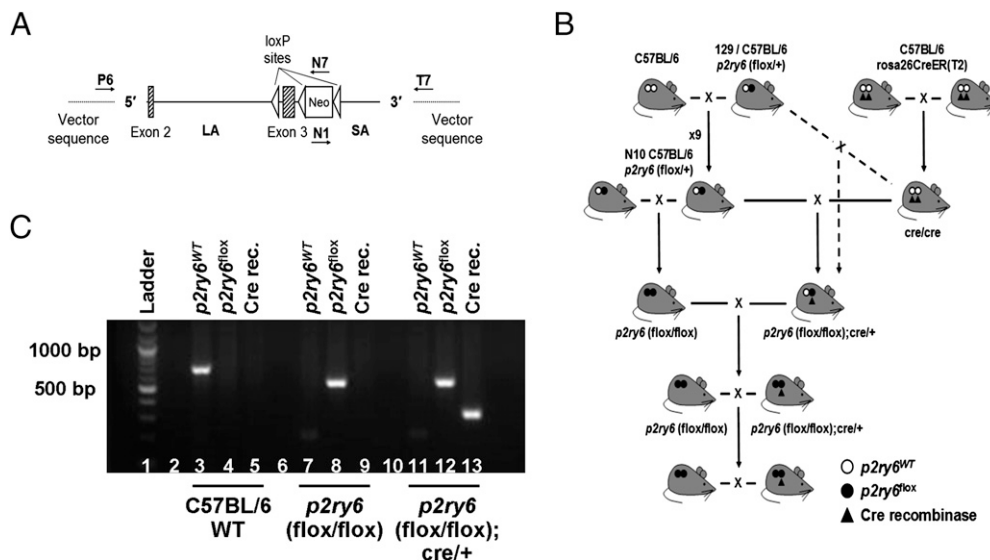


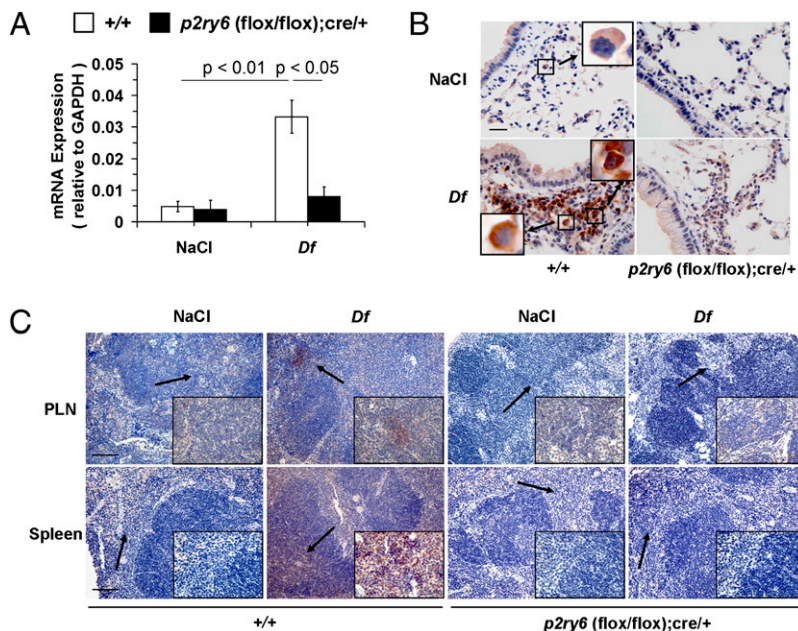
FIGURE 1. Generation of $+/+$ and $p2ry6$ (floxed/floxed);cre/+ mice. **A**, The conditional gene-targeting vector was obtained from an ~ 12.7 kb B6 bacterial artificial chromosome subclone and contained an LA (9.6 kb), $p2ry6$ exons 2 and 3, the latter flanked at the 5' side by a single loxP site and at the 3' side by a PKG-neo cassette with loxP and FRT sites, and an SA (2.1 kb). The target region was ~ 1.8 kb and included exon 3. The annealing sites of the primers used to confirm the structure of the vector are indicated as P6, N7, N1, and T7. **B**, Schematic representation of the breeding protocol used to generate $+/+$ and $p2ry6$ (floxed/floxed);cre/+ mice. $p2ry6$ (floxed/floxed) mice on a B6 $\times$ 129 mixed background (dashed line) or on a B6 background (solid line) were initially mated with cre/cre mice to generate $+/+$ and $p2ry6$ (floxed/floxed);cre/+ strains. **C**, PCR amplification products of the wild-type ($p2ry6^{WT}$) and floxed ($p2ry6^{lox}$) $p2ry6$ gene and of the Cre recombinase in B6 wild-type (lanes 3–5), $p2ry6$ (floxed/floxed) (lanes 7–9), and $p2ry6$ (floxed/floxed);cre/+ (lanes 11–13) mice resolved on a 2% agarose gel.

associated secondary lymphoid organs of $+/+$ mice and whether this distribution and the level of receptor expression changed with inflammation. qPCR revealed low constitutive levels of P2Y₆ receptor mRNA in the lungs of naive $+/+$ mice. These levels sharply increased in response to intranasal *Df* (Fig. 2A). Immunohistochemistry using a rabbit polyclonal anti-P2Y₆ Ab (20) revealed that P2Y₆ receptor protein localized predominantly to cells with the morphologic appearance of macrophages in the alveolar and interstitial spaces in saline-treated $+/+$ mice (Fig. 2B). Challenge of $+/+$ mice with *Df* induced the accumulation of mononuclear cells in the BALT of the BVs, which stained strongly for P2Y₆

receptor protein. On the basis of cell size and morphology, these cells appeared to be a mixture of lymphocytes and monocyte-like cells (Fig. 2B). There was negligible staining of the resident epithelial, endothelial, and smooth muscle cells and no significant staining of the infiltrating granulocytes.

Immunostaining of the spleen and PLNs from naive $+/+$ mice revealed negligible staining for P2Y₆ receptor protein. In contrast, distinct populations of P2Y₆ receptor-positive cells appeared in the paracortical zones of the spleen and PLNs from *Df*-treated $+/+$ mice (Fig. 2C) but not in the germinal centers. Neither the lung (Fig. 2B) nor the lymphoid tissues (Fig. 2C) of the saline- or *Df*-

FIGURE 2. Expression of P2Y₆ receptors in the lungs, PLNs and spleen. **A**, qPCR analysis of P2Y₆ receptor mRNA in the lungs of NaCl- and *Df*-treated $+/+$ ($\square$; $n = 6$ and 18, respectively) and $p2ry6$ (floxed/floxed);cre/+ ($\blacksquare$; $n = 6$ and 15) mice. **B**, Immunohistochemical analysis of P2Y₆ receptors in the lungs from $+/+$ and $p2ry6$ (floxed/floxed);cre/+ mice exposed to NaCl and *Df* intranasally. P2Y₆ receptor protein, indicated by the brown staining, was detected on cells with the morphology consistent with macrophages (upper left panel, inset) in the lung of $+/+$ naive mice and on both macrophages (lower left panel, left inset) and lymphocytes (right inset) in the BALTs of $+/+$ mice. No staining was detected in the lung of NaCl- and *Df*-treated $p2ry6$ (floxed/floxed);cre/+ mice (right panels). Scale bar, 25 μ m. **C**, Immunohistochemistry of P2Y₆ receptors in PLNs and spleen of NaCl- and *Df*-treated $+/+$ and $p2ry6$ (floxed/floxed);cre/+ mice. P2Y₆ receptors were detected on cells located in paracortical T cell-dependent areas of the organs (arrows), as shown in the insets at a higher magnification. Scale bars, 100 μ m. Values in **A** are mean $\pm$ SEM from three independent experiments. Photomicrographs in **B** and **C** are from one representative mouse per group from one of two independent experiments with similar results. Original magnification $\times 63$ and $\times 400$ (insets) (**B**); $\times 20$ and $\times 63$ (insets) (**C**).



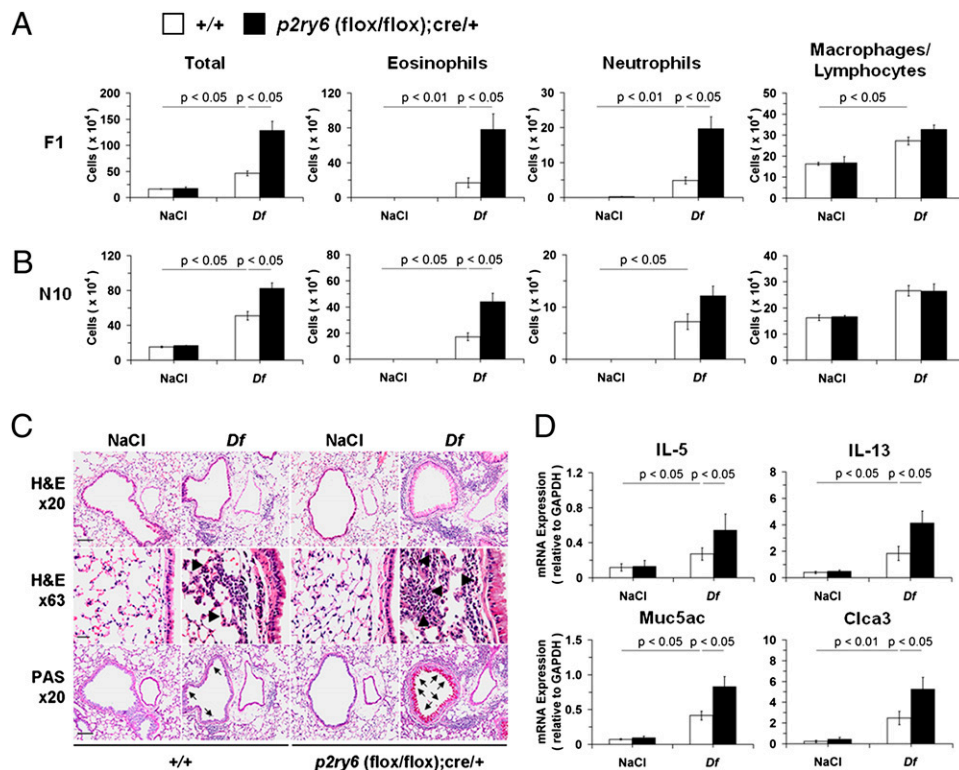


FIGURE 3. *Df*-induced pulmonary inflammation in NaCl- and *Df*-treated *+/+* and *p2ry6* (flo/flo);*cre/+* mice. **A**, Total and differential cell counts from BAL fluid of NaCl- and *Df*-treated *+/+* (□; *n* = 9 and 23, respectively) and *p2ry6* (flo/flo);*cre/+* (■; *n* = 9 and 18) mice at the F₁ stage of breeding. **B**, Total and differential cell counts from BAL fluid of NaCl- and *Df*-treated C57BL/6 *+/+* (□; *n* = 5 and 19, respectively) and *p2ry6* (flo/flo);*cre/+* (■; *n* = 5 and 20) mice at the N10 stage. **C**, Tissue sections of lung showing BVBs from NaCl- and *Df*-treated *+/+* and *p2ry6* (flo/flo);*cre/+* mice were stained with H&E for assessing inflammatory cell infiltrates (H&E, original magnification ×20) and demonstrating tissue eosinophils (H&E, original magnification ×63; arrowheads) or by PAS reaction for depicting mucus-secreting cells (PAS, original magnification ×20; arrows). Scale bars, 100 μm (original magnification ×20) or 25 μm (original magnification ×63). **D**, Expression of mRNA encoding cytokines and mucus-associated proteins in the lung tissue of NaCl- and *Df*-treated *+/+* (*n* = 6 and 18, respectively) and *p2ry6* (flo/flo);*cre/+* (*n* = 6 and 15) mice, measured by qPCR. Data are expressed as ratio of the indicated mRNA expression relative to GAPDH. Values are mean ± SEM from four (**A**) and three (**B**) independent experiments. Photomicrographs in **C** are from one representative mouse per group from one of three independent experiments with similar results. Original magnifications ×20 and ×63.

treated *p2ry6* (flo/flo);*cre/+* mice exhibited staining for P2Y₆ receptor protein.

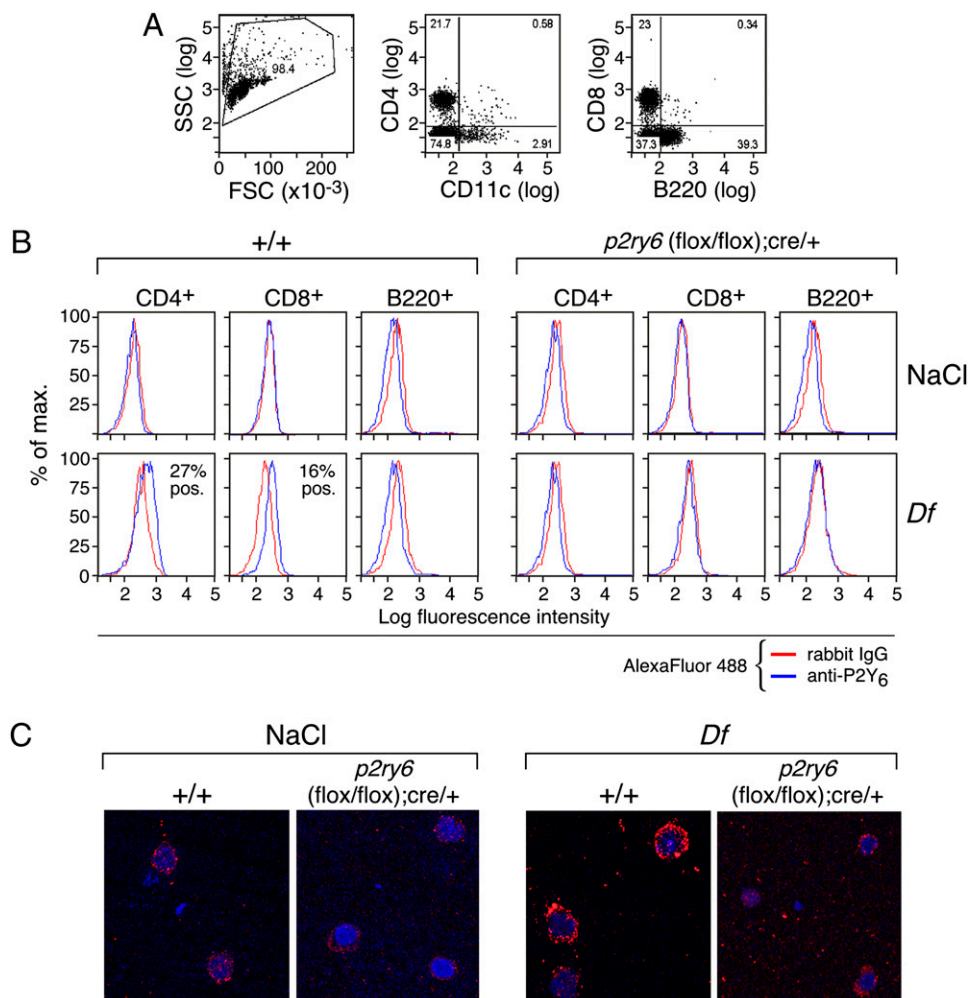
To identify the lymphoid cells expressing P2Y₆ receptors after treatment with *Df*, we performed flow cytometry using the rabbit anti-P2Y₆ Ab on the dispersed spleen cells from the *+/+* mice. Abs against CD4, CD8, B220 (CD45R), and CD11c were used to identify helper and cytotoxic T cells, B cells, and APCs, respectively (Fig. 4A). No P2Y₆-expressing lymphoid cells were identified in the spleens from the naive *+/+* mice (Fig. 4B). In contrast, both CD4⁺ and (to a lesser extent) CD8⁺ splenic T cells from the *Df*-treated *+/+* mice showed modest staining for P2Y₆ (Fig. 4B). B220⁺ B cells did not express P2Y₆ receptors, whether derived from naive or *Df*-treated *+/+* mice. There were too few CD11c⁺ cells to analyze, and no staining for P2Y₆ receptors was observed on any cell populations in the spleens from saline- or *Df*-treated *p2ry6* (flo/flo);*cre/+* mice (Fig. 4B). The percentages of CD4⁺, CD8⁺, and B220⁺ cells in the lymph nodes did not differ between the two strains (data not shown). Confocal imaging of CD4⁺ cells purified from the dispersed lungs of *Df*-treated wild-type mice confirmed prominent P2Y₆ receptor staining, which was not observed in lung T cells from saline-treated *+/+* mice (Fig. 4C).

Effects of P2Y₆ receptors on the adaptive immune response to *Df*

To determine the role of P2Y₆ receptors on control of recall responses to the *Df* Ags in sensitized mice, we evaluated the

production of proinflammatory and immunoregulatory cytokines from restimulated PLN cells from the *Df*-treated *p2ry6* (flo/flo);*cre/+* mice and *+/+* controls. The PLNs from the *Df*-treated *p2ry6* (flo/flo);*cre/+* mice were consistently larger than those from the *+/+* controls and contained more total cells (Fig. 5A). Equal numbers of PLN cells isolated from NaCl- and *Df*-treated *+/+* and *p2ry6* (flo/flo);*cre/+* mice were cultured in vitro in either medium alone or containing *Df*. At the end of the culture, the concentrations of IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ released in the supernatants were evaluated by ELISA. Restimulated PLN cells from *Df*-treated *p2ry6* (flo/flo);*cre/+* mice generated more IL-4, IL-5, IL-13, and IFN-γ than did cells from *+/+* mice (Fig. 5B). The amounts of IL-2, IL-10, and IL-17A also tended to be higher but did not reach significance (*p* = 0.26, 0.15, and 0.12, respectively). The results obtained from the PLN cells from the N10 mice were similar (Supplemental Fig. 3), and restimulation of splenocytes yielded results similar to the PLN cells (data not shown). To determine whether the increased quantities of cytokines reflected increased numbers of cytokine-generating cells in the PLNs from *Df*-treated *p2ry6* (flo/flo);*cre/+* mice, we performed ELISPOT assays. Consistent with the ELISA data, the PLNs of *Df*-treated *p2ry6* (flo/flo);*cre/+* mice contained increased numbers of cells expressing IL-4, IL-5, and IL-13 protein than did the PLNs of the *Df*-treated *+/+* controls (Fig. 5C). The differences in IFN-γ and IL-17A were not significant (*p* = 0.07 and 0.10, respectively). Quantitative PCR analysis of the *Df*-treated PLN cells showed no differences between strains in the

FIGURE 4. Cytofluorographic detection of P2Y₆ receptors on PLN cell subsets. *A*, Dot plots showing the forward light scatter (FSC)/side scatter (SSC)-gated cells and the analysis of the expression of CD4, CD11c, CD8, and B220 on PLN cells from *Df*-treated *+/+* mice. Percentages of positive and negative cells are displayed. *B*, Histograms showing the expression of P2Y₆ receptors (blue lines) versus isotype control (red lines) on CD4⁺, CD8⁺, and B220⁺-gated PLN cells in NaCl- and *Df*-treated *+/+* and *p2ry6* (flox/flox);cre/+ mice. Percent positives are displayed. *C*, Confocal images of CD4⁺ cells purified from the lungs of the indicated NaCl- or *Df*-challenged strains. Panels are from one experiment representative of two with similar results. Original magnification $\times 400$.



induced expressions of the Th1-associated transcription factor T-bet, the Th2-associated transcription factor GATA-3 (the regulatory T cell-associated transcription factor Foxp3), and the Th17 transcription factor ROR- γ t (Supplemental Fig. 4).

To determine whether the absence of P2Y₆ receptors changed the rate of helper T cell proliferation, PLN cells were loaded with CFDA-SE before restimulation, and the dilution of the dye was measured cytofluorographically as an index of proliferation.

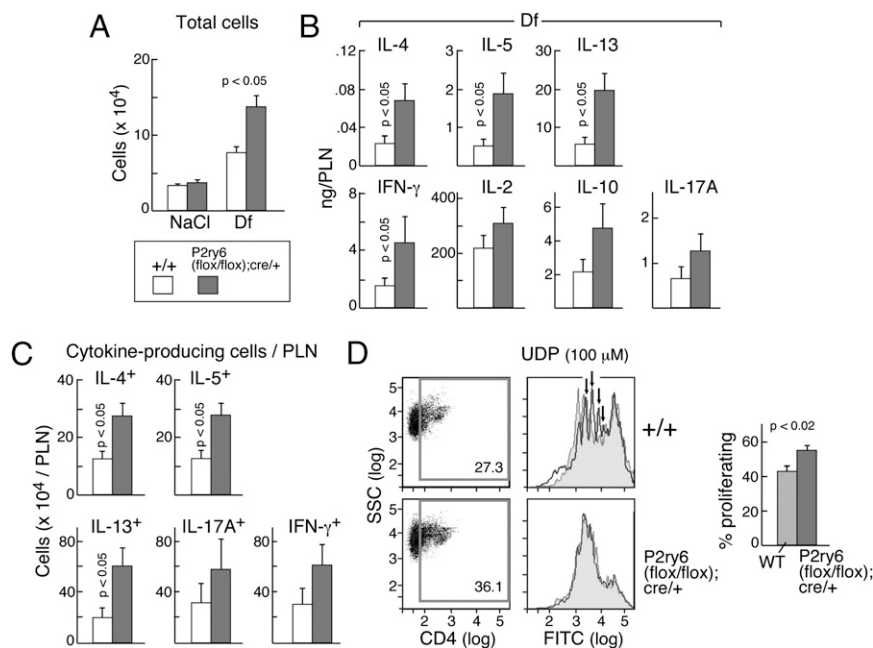


FIGURE 5. PLN cellularity and T cell responses. *A*, PLN cell numbers from NaCl- and *Df*-treated *+/+* ($\square$; $n = 5$ and 17, respectively) and *p2ry6* (flox/flox);cre/+ ($\blacksquare$; $n = 5$ and 17) mice. *B*, Cytokine release from restimulated PLN cells of *Df*-treated *+/+* ($n = 16$) and *p2ry6* (flox/flox);cre/+ ($n = 17$) mice. *C*, Number of cytokine-producing cells in PLN cells of *Df*-treated *+/+* ($n = 15$) and *p2ry6* (flox/flox);cre/+ ($n = 13$) mice. Values in A–C are mean $\pm$ SEM from three independent experiments. *D*, Proliferation (based on CFDA-SE dilution) of CD4⁺-gated PLN cells from *Df*-treated *+/+* and *p2ry6* (flox/flox);cre/+ mice (N10 generation) cultured with plate-bound anti-CD3 and soluble anti-CD28 Abs. The mean $\pm$ SEM percentage of proliferating cells from five mice per group are shown (right panels). Histograms showing the CFSE dilution of the CD4⁺ gate of polyclonally stimulated spleen cells treated with UDP (100 μ M, black) are overlaid with histograms of untreated cells (gray) from the same strains in one experiment representative of two performed.

Because the CFDA-SE dilution signal in cultures of PLN cells restimulated with *Df* was below the limit of detection (data not shown), we performed the assay by using a T cell polyclonal stimulation with anti-CD3 and anti-CD28 Abs. Percentages of polyclonally stimulated proliferating CD4⁺ T cells were modestly but significantly higher in the PLNs of *p2ry6* (flox/flox);cre/+ mice than in the PLNs of +/+ controls (Fig. 5D). Splenic CD4⁺ cells *p2ry6* (flox/flox);cre/+ mice also displayed higher rates of proliferation in response to polyclonal stimulation than did +/+ control cells, and unlike the +/+ cells, exogenous UDP failed to suppress their proliferation (Fig. 5D). Percentages of apoptotic CD4 cells in the PLNs of the *Df*-treated *p2ry6* (flox/flox);cre/+ mice were lower than in the PLNs from the +/+ controls (Supplemental Fig. 5). The *p2ry6* (flox/flox);cre/+ mice exposed to *Df* showed higher levels of serum total IgE (Supplemental Fig. 6A), but they had comparable levels of *Df*-IgG₁ (Supplemental Fig. 6B).

The absence of P2Y₆ receptors on CD4⁺ T cells controls the recall response to Df-associated allergens

To determine whether the increased cytokine production from the restimulated PLNs reflected a direct suppressive effect of P2Y₆ receptors expressed by T cells or an effect of P2Y₆ receptor-dependent modulation of APC functions, we developed a co-

culture assay. Because mice with transgenic expression of *Df*-specific TCR are not yet available, we purified CD3⁺/CD4⁺ T cells from the spleens of NaCl- and *Df*-treated +/+ and *p2ry6* (flox/flox);cre/+ mice and incubated them with enriched interstitial macrophages from the lungs of each genotype and treatment as a source of APCs. Nearly all of these cells had morphologic features of macrophages (Fig. 6A), with >85% expressing the pan-macrophage marker CD68 (Fig. 6B) (37). Fewer than 5% of the cells expressed T cell markers (CD3, CD4, and CD8), whereas B220 was expressed by ~15% of the CD68⁺ cells (data not shown). The proportions of the cells expressing the APC markers CD11c and MHC-II (~30% for each marker) did not differ between genotypes (Fig. 6B), and confocal imaging confirmed that the macrophages from the *Df*-treated +/+ mice exhibited substantially increased expression of P2Y₆ protein relative to the saline-treated controls (Fig. 6C). After 72 h of coculture in the presence of *Df*, CD3⁺/CD4⁺ T cells from naive mice of both genotypes generated small and comparable amounts of IL-5 and IL-13 (Fig. 6, left panels). In contrast, CD3⁺/CD4⁺ T cells from *Df*-treated *p2ry6* (flox/flox);cre/+ mice released more IL-5, IL-13, and IFN- γ than the cells from +/+ mice in all three experiments performed (as shown for one experiment; Fig. 6D, right panels). There was no effect of the macrophage genotype, although macrophages from lungs of *Df*-treated mice supported higher levels of

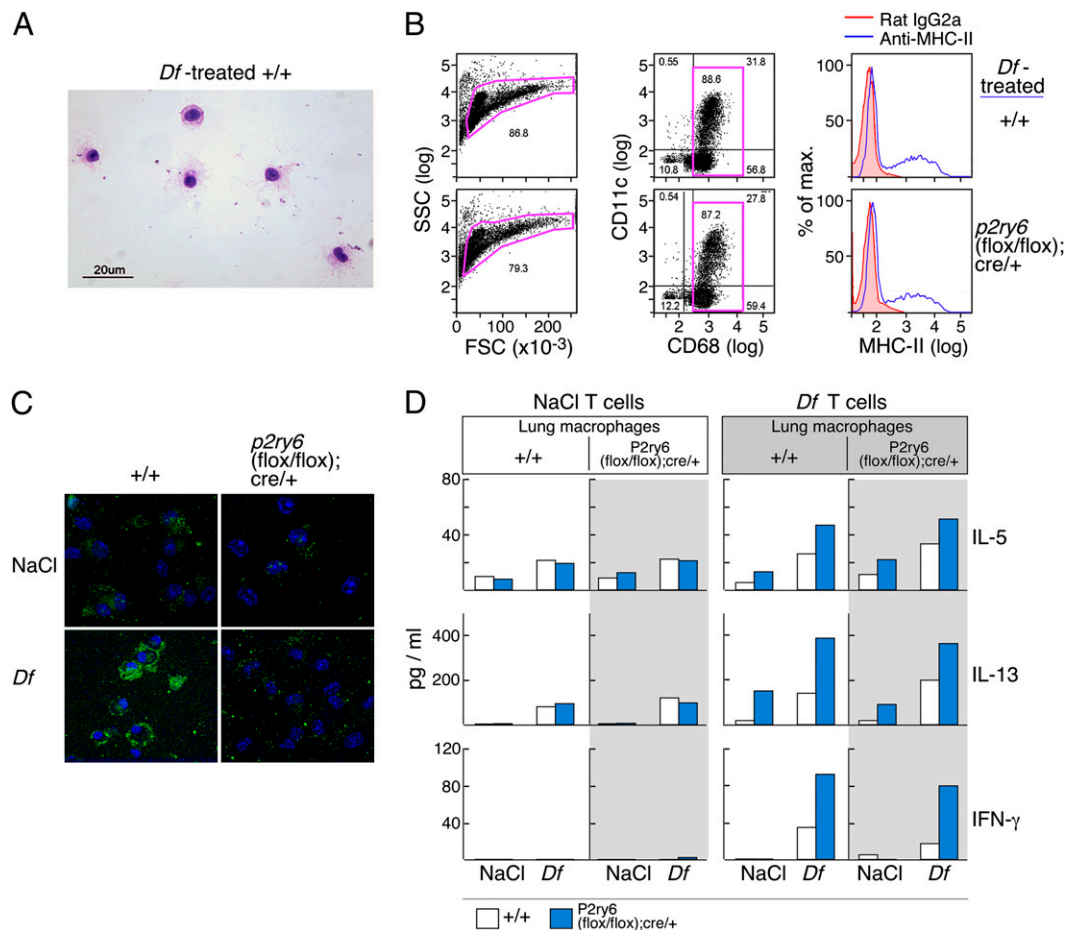


FIGURE 6. Cytokine release from restimulated cocultures of CD4⁺ T cells and lung macrophages. *A*, Wrights and Giemsa stain showing enriched lung macrophages from *Df*-treated +/+ mice. *B*, Forward and side scatter characteristics (*left panels*), CD68/CD11c staining (*middle panels*), and MHC-II staining (*right panels*) of the enriched macrophages from the indicated strains used in the coculture assays. *C*, Confocal images of enriched lung APCs from the indicated NaCl- and *Df*-treated genotypes stained for P2Y₆ immunofluorescence. Results were identical in a second experiment. *D*, Cytokine release from restimulated cocultures of CD4⁺ T cells and lung macrophages. Lung macrophages from NaCl- and *Df*-treated +/+ (unshaded) and *p2ry6* (flox/flox);cre/+ (shaded) mice were cocultured with CD4⁺ T cells from +/+ (□) and *p2ry6* (flox/flox);cre/+ (■) mice treated with NaCl (*left panels*) or *Df* (*right panels*). The results are from one experiment, which was repeated twice more with similar trends but with different magnitude of responses.

cytokine generation by CD4⁺ cells than did macrophages from the lungs of saline-treated controls (Fig. 6D).

Discussion

This study uncovers a prominent role for the UDP-selective P2Y₆ receptor in the control of pulmonary inflammation and cytokine generation in a model of allergic pulmonary inflammation. In our model, uracil nucleotides act at P2Y₆ receptors that are inducibly expressed by T cells to suppress the development of *Df* Ag-responsive T cells and production of pathogenetic cytokines. In vitro studies indicate that UDP (acting presumably at P2Y₆ receptors) amplifies effector cytokine production by epithelium (21), endothelium (28), macrophages (27), and MCs (20). Although we cannot exclude important contributions from P2Y₆ receptors on these other cell types in our model, our study suggests that upregulation of P2Y₆ receptors on T cells and limiting T cell activation may be a dominant mechanism that limits the pathologic consequences of a pulmonary immune response, at least in response to a complex natural allergen.

Given the fact that multiple P2Y₆ receptor-expressing cell types can contribute to the pathophysiology of the immune and inflammatory response to natural allergens, we tested the effect of P2Y₆ receptor deletion in a model of intranasal *Df* challenge. *Df* contains glycans, proteases, and other stimuli that activate lung APCs and initiate a pathogenetic T cell response with the generation of Th1, Th2, and Th17 cytokines and the development of characteristic pulmonary inflammation (38, 39). We generated a mouse with homozygous floxed *p2ry6* alleles (Fig. 1A) that were disrupted by tamoxifen treatment after introducing a single copy of a tamoxifen-sensitive Cre recombinase allele [*p2ry6* (floxed/floxed); cre/+] (Fig. 1B, 1C). These mice were fertile, healthy, and indistinguishable from littermate +/+ controls, similar to a previously reported conditional *p2ry6*^{-/-} mice (16). On the basis of the role for P2Y₆ receptors in amplifying cytokine production by immune cells in vitro, we anticipated that the deletion of this receptor might reduce inflammation in our model. We were surprised to find that the *p2ry6* (floxed/floxed); cre/+ mice exhibited increased inflammation after *Df* administration compared with the +/+ littermates, showing significantly increased total cell counts, increased eosinophil and neutrophil counts in the BAL fluid (Fig. 3A, 3B), as well as increased bronchovascular cellular infiltration and goblet cell metaplasia on histological analysis (Fig. 3C). The increases in goblet cell-associated transcripts in the lungs of *p2ry6* (floxed/floxed); cre/+ mice, which are characteristically controlled by the Th2 cytokine IL-13 (40–42), was paralleled by increased quantities of transcripts encoding IL-13, as well as IL-5, the major cytokine responsible for eosinophilia (Fig. 3D) (43, 44). Thus, the absence of P2Y₆ receptors increased the expression of pathogenetic Th2 cytokines in the target tissue, leading to characteristic lung pathology.

Although cytokine generation in models of allergen-induced pulmonary disease requires T cell activation following the presentation of Ags by APCs (39), several additional cell types, including epithelial cells, modify cytokine generation by recognizing nonprotein components of dust mites (45–47). We determined the distribution of P2Y₆ receptor expression in resident lung cells in both naive and *Df*-treated mice. P2Y₆ receptor staining in the lungs of naive +/+ mice was restricted primarily to macrophages in both the alveolar and interstitial spaces of the lung (Fig. 2B). The sharply increased expression of P2Y₆ receptor transcript in the lungs of *Df*-treated +/+ mice (Fig. 2A) was paralleled by the appearance of monocytoid and lymphocytoid cells that strongly expressed P2Y₆ receptor protein and that localized to the BALT (Fig. 2B). In addition, staining of the regional lymph nodes and of

the spleen suggested that *Df* treatment of +/+ mice induced a P2Y₆ receptor-expressing T cell population (Fig. 2C), and flow cytometry verified that PLN CD4⁺ cells (and, to a lesser extent, CD8⁺ cells) expressed P2Y₆ receptors after exposure in vivo to allergen (a pattern recapitulated by lung T cells; Fig. 4). The absence of P2Y₆ receptor expression on B220⁺ B cells is consistent with the lack of immunostaining of germinal centers (Fig. 2C). Because there were very few CD11c⁺ APCs in the PLNs, we were unable to determine whether they expressed P2Y₆ receptors, although macrophages enriched from the lung did express P2Y₆ receptors that were upregulated with *Df* treatment (Fig. 6C).

We next sought to determine the effects of P2Y₆ receptor deletion on the recall response to *Df*. The PLNs from the *Df*-treated *p2ry6* (floxed/floxed); cre/+ mice contained more total cells (Fig. 5A) than did the PLNs from the +/+ controls. Although the total percentages of T cells in the PLNs did not differ between the strains (data not shown), the restimulated PLN cells from the *Df*-treated *p2ry6* (floxed/floxed); cre/+ mice generated significantly higher amounts of Th2 and Th1 cytokines than did samples from the +/+ mice (Fig. 5B), paralleled by higher numbers of cytokine-producing T cells (Fig. 5C). Thus, the effect of P2Y₆ receptors on the control of Ag recall responses is not restricted to one lineage of effector T cell in this model. Moreover, the robust generation of IL-10 (Fig. 5B) by the samples from *p2ry6* (floxed/floxed); cre/+ mice and the trivial induction of mRNA encoding Foxp3 in PLN cells from +/+ and *p2ry6* (floxed/floxed); cre/+ mice following exposure to *Df* (Supplemental Fig. 4) suggest that the absence of P2Y₆ receptors does not selectively compromise regulatory T cell development as a mechanism for the phenotype observed. Thus, P2Y₆ receptors induced by Ag stimulation play a homeostatic role in the control of memory T cell activation during recall responses to *Df* Ags. Although the low frequencies of *Df*-responsive T cells (estimated at ~1 in 100 lymph node cells) precluded detection of proliferation in a recall response to *Df*, polyclonally stimulated CD4⁺ PLN T cells from *p2ry6* (floxed/floxed); cre/+ mice proliferated significantly faster than +/+ control cells did. The fact that exogenous UDP suppressed proliferation only in the CD4⁺ cells from +/+ controls (Fig. 5D) validates that P2Y₆ receptors account entirely for the UDP response of T cells in this model. It is possible that both enhanced proliferation and attenuated apoptosis contribute to the relative PLN hyperplasia of the *p2ry6* (floxed/floxed); cre/+ mice in this model. Proof of a homeostatic role for P2Y₆ in the control of Ag-induced proliferation awaits the creation of transgenic mice with TCRs specific for *Df* Ags.

In addition to intrinsic properties of the responder T cells, APC-specific factors strongly influence the magnitude and nature of Ag-induced recall responses (39). To test whether the outcome of the *Df*-induced recall response was influenced more strongly by P2Y₆ receptors expressed by APCs or by effector T cells, we purified P2Y₆ receptor-deficient or -sufficient splenic CD4⁺ T cells and cocultured these with either P2Y₆ receptor-deficient or -sufficient lung macrophages as a source of APCs (Fig. 6) (48). The induction of inflammation by *Df* causes APCs to mature and become more effective in processing and presenting Ag, likely explaining the ability of the CD4⁺ cells to produce higher level of cytokines when cocultured with APCs derived from *Df*-treated mice. The absence of P2Y₆ receptors did not affect the expression of activated APC markers CD11c and MHC-II (Fig. 6). Importantly, the macrophage genotype had no influence on the levels of cytokines generated by responder T cells. Although the absolute quantities of cytokines produced varied in the three experiments performed, CD4⁺ cells from the *Df*-treated *p2ry6* (floxed/floxed); cre/+ mice generated more IL-5, IL-13, and IFN-γ than did the CD4⁺ T cells from the +/+ controls in every experiment (as shown for one

experiment; Fig. 6D). Thus, at least at the effector phase of the *Df*-induced immune response, the absence of P2Y₆ on *Df*-treated CD4⁺ cells is both necessary and sufficient to amplify the production of cytokines in response to stimulation with the allergen in a recall response.

To our knowledge, our study demonstrates the first homeostatic role of P2Y₆ receptors in the intrinsic control of effector T cell responses to clinically relevant allergens. The P2Y₆ receptor mediates a suppressive function for the uracil nucleotides that are generated in the inflammatory milieu during exposure to dust mite allergens. Two recent studies reported that uridine, the precursor of UDP and UTP, has a protective function in two models of acute lung inflammation by acting on epithelial cells (49, 50). Thus, uracil and its derivatives may suppress allergic pulmonary inflammation by several mechanisms, contrasting with the proinflammatory effects of adenine nucleotides in a similar context. Given the multitude of P2Y receptors that are expressed by cells of the immune system, it is critical to understand both the pro- and anti-inflammatory effects of any P2Y receptor that is envisioned as a potential target for drug development in the treatment of inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure Legends

Supplemental Figure 1. *Df*-induced pulmonary inflammation and PLN cellularity and cytokine release in NaCl- and *Df*-treated C57BL/6 WT and *cre/+* mice

(A) Total and differential cell counts from BAL fluid of NaCl- and *Df*-treated C57BL/6 WT (open bars; $n = 8$ and 17 , respectively) and *cre/+* (filled bars; $n = 7$ and 18) mice. (B) Number of cells obtained from PLNs of NaCl- and *Df*-treated C57BL/6 WT and *cre/+* mice. (C) Cytokine release from restimulated PLN cells of *Df*-treated C57BL/6 WT and *cre/+* mice.

Values are mean $\pm$ SEM from two independent experiments.

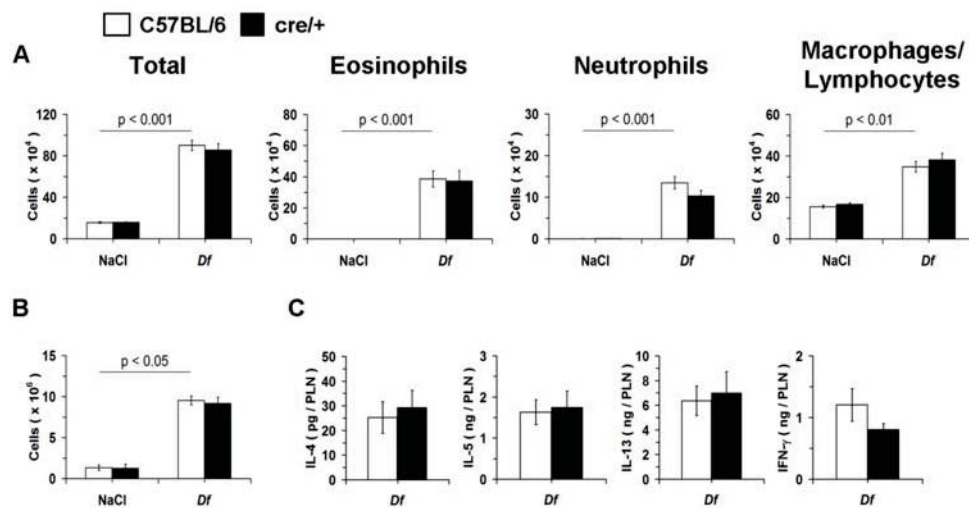
Supplemental Figure 2. Quantitative PCR analysis of mRNA encoding chemokines CCL11, CXCL1, and CCL2 from the lungs of NaCl- and *Df*-treated mice from the indicated genotypes. Results are the mean $\pm$ SEM from 5 mice/group from a single experiment representative of two performed.

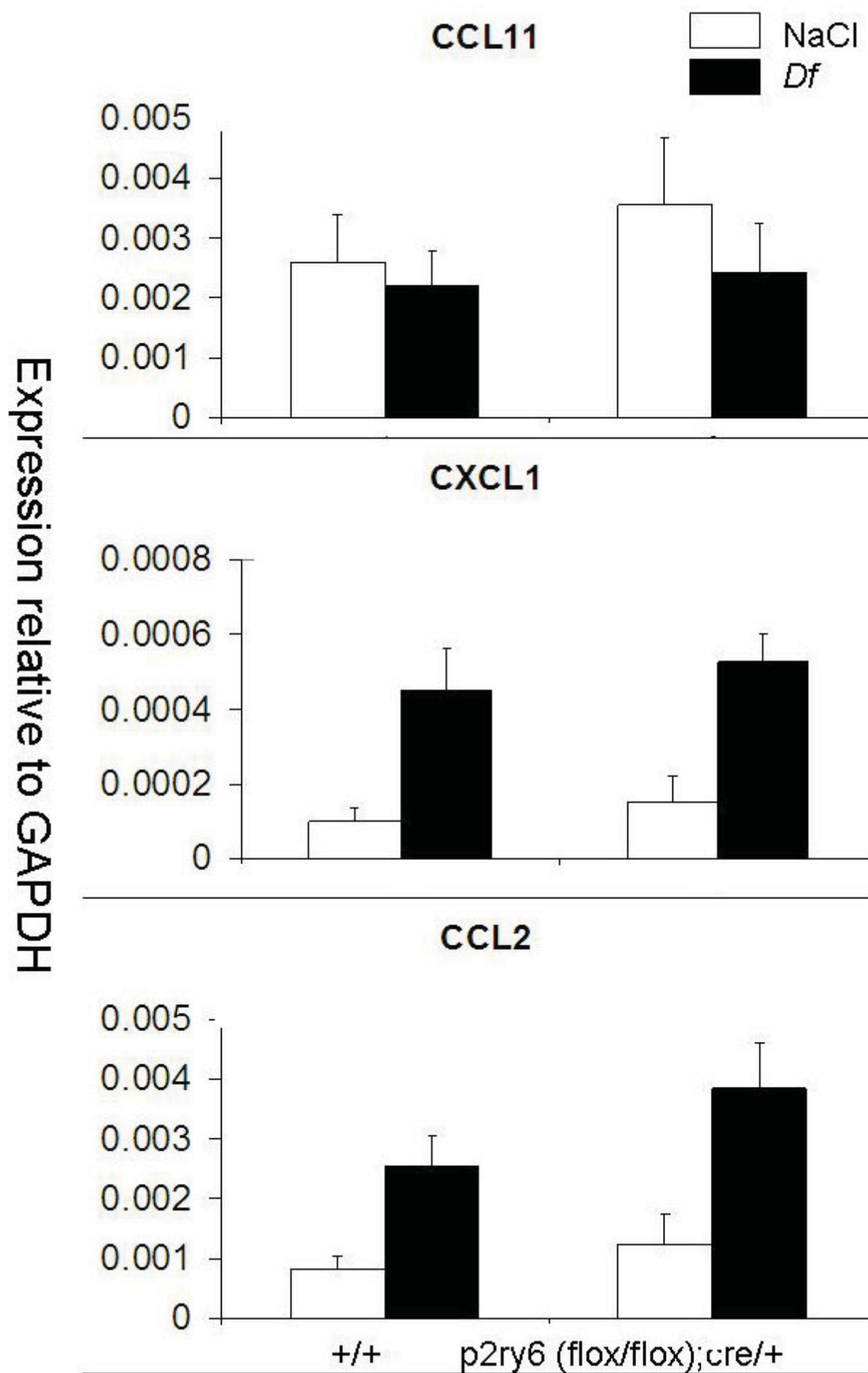
Supplemental Figure 3. Production of the indicated cytokines by the *Df*-restimulated PLN cells from *p2ry6* (flox/flox); *cre/+* mice and *+/+* controls backcrossed for 10 generations and subjected to *Df* challenge. Results are mean $\pm$ SD from 9-12 mice per group from two different experiments.

Supplemental Figure 4. Quantitative PCR analysis of transcription factor expression by PLN cells from the indicated strains of *Df*-treated mice. Results are mean $\pm$ SD from 10 mice in each group.

Supplemental Figure 5. Annexin V staining of CD4⁺ T cells in the PLN from NaCl- or Df-challenged mice from the indicated genotypes. Gray histogram is unstained cells. The proportion of Annexin V-positive cells in the CD4⁺ gate from pooled PLN cells from 5 mice/group is shown for a single experiment. Results in a second experiment were similar.

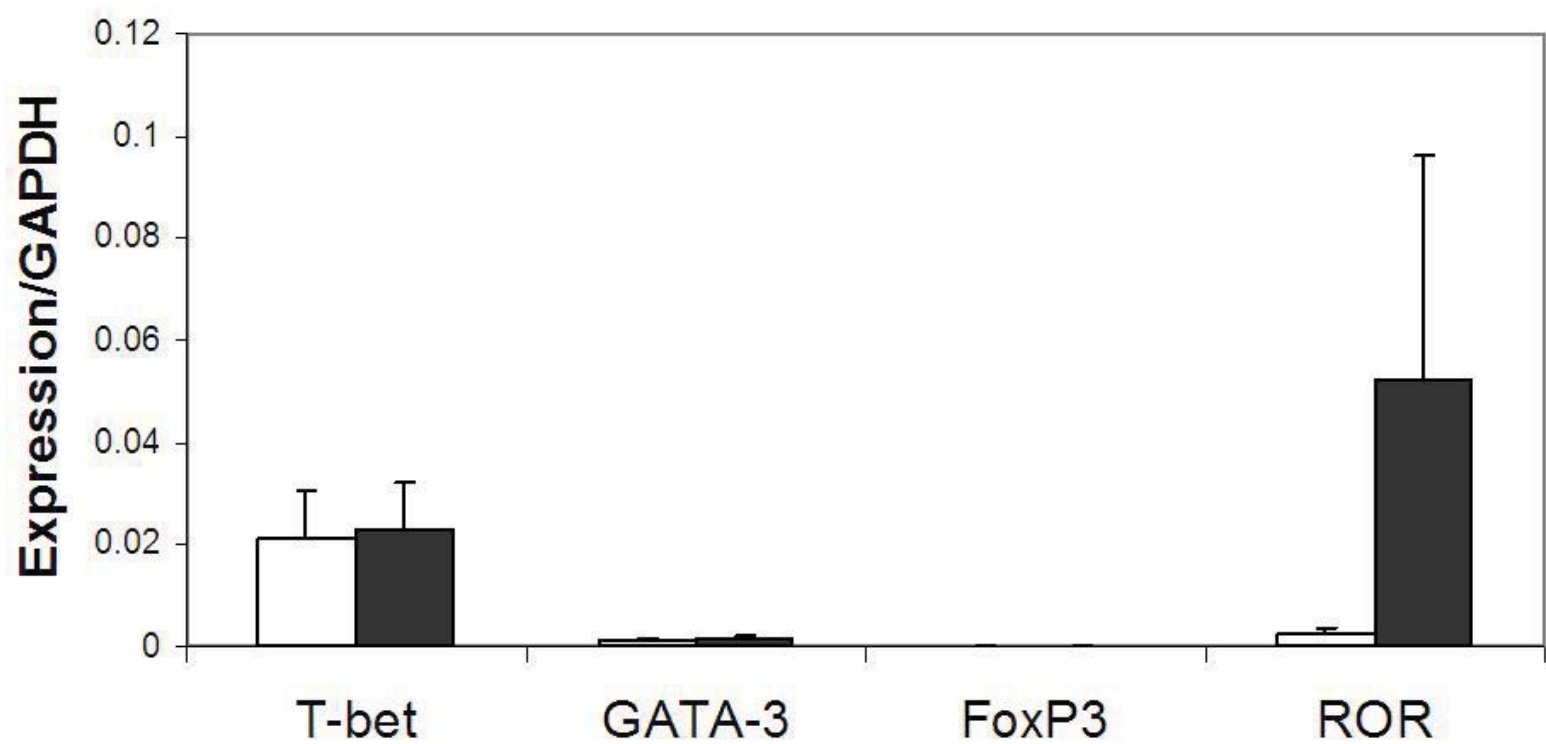
Supplemental Figure 6. Immunoglobulin levels in serum of NaCl- and *Df*-treated *+/+* and *p2ry6* (*flox/flox*); *cre/+* mice. Results are from 5 mice in each group.





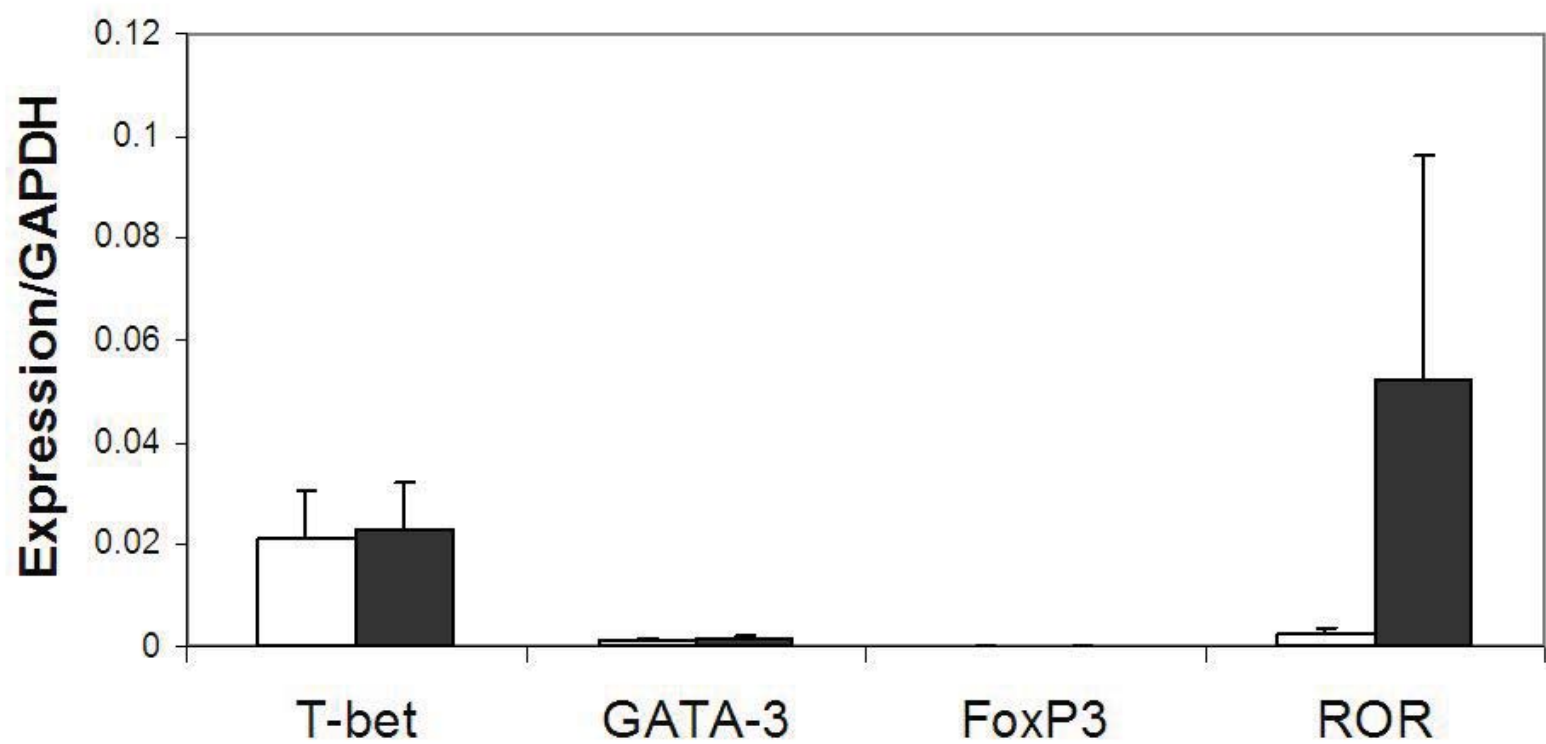
□ **+/+** ■ ***p2ry6* (flox/flox);cre/+**

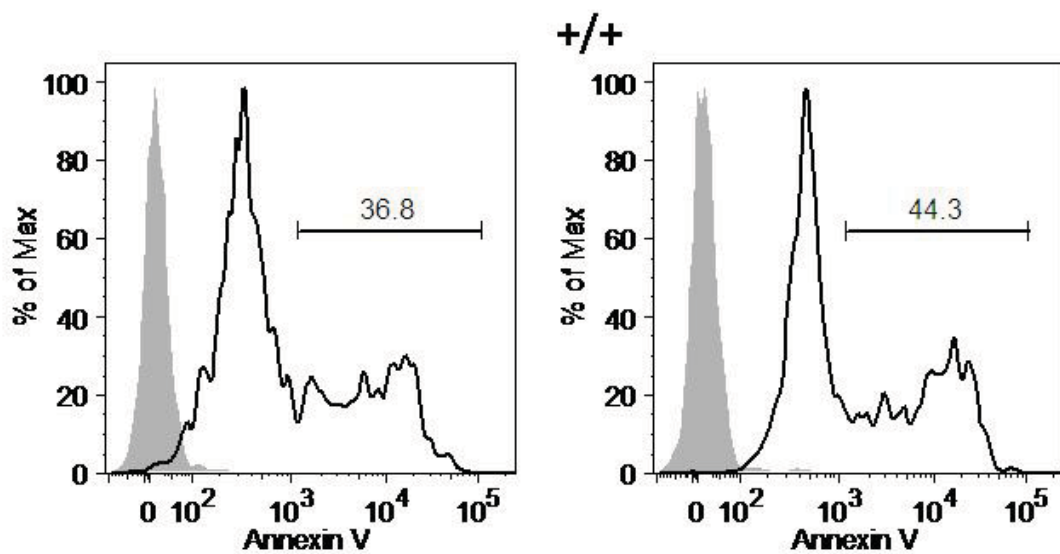
Transcription factor PCR



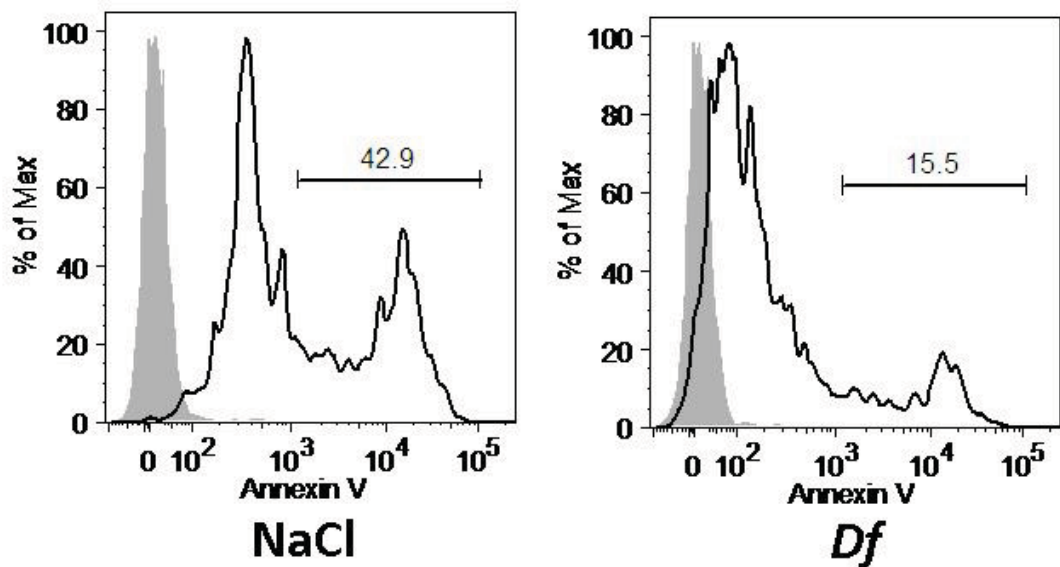
□ **+/+** ■ ***p2ry6* (flox/flox);cre/+**

Transcription factor PCR



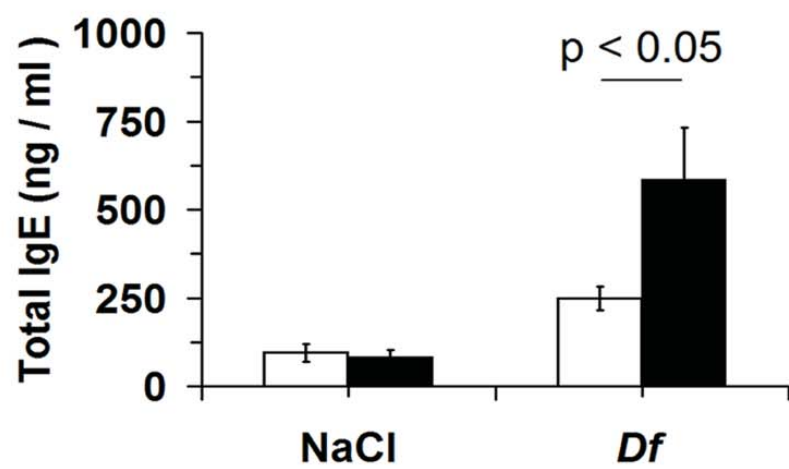


P2ry6 (flox/flox);cre/+



□ +/+ ■ *p2ry6* (flox/flox);cre/+

A



B

