

Naive T cells sense the cysteine protease allergen papain through protease-activated receptor 2 and propel T_H2 immunity

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Background: Sensitization to protease allergens, such as papain, or helminth infection is associated with basophil recruitment to draining lymph nodes (LNs). Basophils have the capacity to present antigen to naive T cells and promote T_H2 differentiation directly or indirectly through IL-4 production.

Objective: We studied how papain induces basophil migration to LNs and the contribution of various leukocytes to papain-induced immune responses.

Methods: We immunized mice in the footpad with papain and studied leukocyte recruitment and inflammatory cytokine and chemokine production in the draining popliteal LNs.

Results: Papain directly activated naive T cells through protease-activated receptor (PAR) 2 to initiate a chemokine/cytokine program that includes CCL17, CCL22, and IL-4. Papain-triggered innate immune responses were dependent on both CD4 T cells and PAR2 and were strongly reduced in the absence of CCR4, the primary receptor for CCL17/CCL22.

Conclusion: These results elucidate a novel innate allergen-recognition pathway mediated by naive T cells through PAR2, which provide an immediate source of chemokines and IL-4 upstream of basophils and antigen-restricted T_H2 differentiation. PAR2 antagonism might thus hold promise for the treatment of allergic disease. (*J Allergy Clin Immunol* 2012;■■■■:■■■-■■■.)

Key words: Chemokines, basophils, chemotaxis, T_H2, allergens

The plasticity and diversity of specific T-lymphocyte populations shape the adaptive immune response. Naive T cells differentiate into IFN- γ -producing T_H1 cells after infection with intracellular microbes, including viruses and protozoa.¹ T_H2

Abbreviations used

APC: Antigen-presenting cell
BM: Bone marrow
BMB: Bone marrow-derived basophil
DC: Dendritic cell
FITC: Fluorescein isothiocyanate
Jak: Janus kinase
LN: Lymph node
MC: Mast cell
OVA: Ovalbumin
PAR: Protease-activated receptor
PE: Phycoerythrin
pLN: Popliteal lymph node
qPCR: Quantitative PCR
STAT: Signal transducer and activator of transcription
WT: Wild-type

cells, which secrete IL-4, IL-5, and IL-13, develop in response to helminth infection and allergen exposure. The pathways linking the innate and adaptive response to allergens, in which antigen-presenting cells (APCs) recognize allergens and induce antigen-specific effector T_H2 differentiation, have not been fully defined.^{2,3}

Many allergens have intrinsic proteolytic activity,⁴ which is required for their induction of T_H2-mediated allergic inflammation.³ Papain is a cysteine protease belonging to a superfamily of pathogen-derived proteases, including gingipains and cruzipain of *Trypanosoma cruzi*.⁵ Papain can elicit antigen-specific IgE production and degranulation of mast cells (MCs) and is associated with occupational allergy in food industry workers and latex sensitivity caused by allergen cross-reactivity.^{6,7} Published work has suggested a function for basophils in the murine T_H2 immune response to allergens, including papain. Subcutaneous injection of papain into mice, either in the presence^{8,9} or absence^{10,11} of a “bystander” antigen, such as ovalbumin (OVA), elicited basophil accumulation in draining lymph nodes (LNs). Published studies have also provided evidence that basophils regulate T_H2-mediated immunity to helminths,¹² tick antigens,¹³ and antigen-IgE complexes¹⁴ in mice.

Basophils are short-lived leukocytes that circulate in the bloodstream and migrate transiently into the T-cell zone of draining LNs after papain immunization.^{10,11} The chemokines and receptors guiding basophil trafficking in mice are unknown. We examined the LN inflammatory response after subcutaneous papain immunization. Basophils were drawn to and CCL17 and CCL22 accumulated in popliteal lymph nodes (pLNs) of mice

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exposed to papain compared with PBS-treated LNs, none of which was observed when *Cd4*^{-/-} or *Par2*^{-/-} mice were studied. Exposure of naive T cells to papain *in vitro* elicited production of CCL17/CCL22 and IL-4. These results illuminate a novel innate function of naive T cells and define basophil-trafficking pathways involved in the immune response to a protease allergen.

METHODS

For additional details on methods, see the Methods section in this article's Online Repository at www.jacionline.org.

Mice

Wild-type (WT) C57BL/6, *Cd4*^{-/-}, *Tcrα*^{-/-}, *Ccr4*^{-/-}, *Cd11c-Dtr-egfp*, and *Par2*^{-/-} mice (all on a C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, Me). DO11.10/4get mice on a *Rag1*^{-/-} background were provided by Dr Markus Mohrs (Trudeau Institute, Saranac Lake, NY). All murine experiments were performed in accordance with Animal Study Protocol LAD-3E, which was approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee, National Institutes of Health.

Bone marrow cultures, sorting, and chemotaxis

Whole bone marrow (BM) cells were obtained from murine femurs and cultured for 10 days in RPMI medium supplemented with IL-3 (30 ng/mL), 10% FBS, 50 μmol/L 2-mercaptoethanol, and 2 mmol/L L-glutamine. FcεRI⁺CD49b⁺c-kit⁻ cells were sorted with a FACSARIA Cyan followed by recovery for 24 hours in culture medium before chemotaxis assays. In some assays bone marrow–derived basophils (BMBs) were enriched from BM cultures by using CD49b microbeads alone (Miltenyi Biotec, Bergisch Gladbach, Germany), which produced results equivalent to those observed with BMBs sorted by means of flow cytometry. Chemotaxis assays were performed with 5-μm pore size 24-well ChemoTx system (Costar, Cambridge, Mass) per the manufacturer's instructions. Cells were allowed to migrate in the presence of chemokine in the lower chamber for 4 hours followed by quantification by means of hemocytometry.

Statistical analysis

Data are presented as means ± SEMs where applicable and were analyzed by using 1- or 2-way ANOVA or the Student *t* test by using PRISM software (GraphPad Software, Inc, La Jolla, Calif). We considered a *P* value of less than .05 to be statistically significant.

RESULTS

Inflammatory response of LNs to papain immunization

To examine papain-evoked pathways, we injected mice subcutaneously in the rear footpad with either PBS or papain and then harvested the draining (popliteal) LNs 2 to 4 days after immunization (see Fig E1 in this article's Online Repository at www.jacionline.org). Consistent with published studies,^{10,11} we observed an accumulation of basophils (FcεRI⁺CD49b⁺c-kit⁻) in the pLNs 3 days after papain injection but not in the pLNs of mice injected with PBS (see Fig E1, A). We also found an increased frequency of CD4⁺IL-4⁺ T cells after restimulation of pLNs of papain-injected mice 4 days after immunization (see Fig E1, B) and serum papain-specific IgE, which peaked 14 days after immunization (see Fig E1, C). These results indicated a T_H2-mediated immune response to papain.

We compared chemokine/cytokine gene expression in the pLNs of PBS- or papain-immunized mice 2 days after injection using a quantitative PCR (qPCR) array. Several transcripts were significantly upregulated in the LNs of papain-immunized mice compared with those of PBS-treated mice, most notably *Ccl17* (5-fold), *Ccl22* (3.5-fold), *Ccl6* (3-fold), and *Il4* (3-fold; Fig 1, A). By using real-time PCR, we found that expression of both *Ccl17* and *Ccl22* was significantly increased in the pLNs 2 days after papain injection compared with that seen in PBS-treated pLNs (Fig 1, B). In pLN homogenates quantities of immunoreactive CCL17 and CCL22 were increased significantly (7- and 3-fold, respectively) in papain-treated mice compared with that seen in PBS-immunized mice (Fig 1, C).

Basophil chemokine receptor expression and chemotaxis

To determine relevant papain-elicited chemokines that attract basophils to the draining LNs, we analyzed chemokine receptor expression on basophils isolated from the spleen and BM or on basophils derived from BM precursors cultured in the presence of IL-3 for 7 to 10 days (BMBs; see Fig E2, A in this article's Online Repository at www.jacionline.org). Freshly isolated BM and splenic basophils expressed CCR2 (Fig 2, A and B) but not CCR3 (see Fig E2, B). We also detected expression of CCR4 and CXCR2 (Fig 2, A and B) but no or minimal CCR1, CCR5, CCR6, CCR7, CXCR4, and CXCR5 (see Fig E2, B). Although chemokine receptor expression on BMBs was qualitatively similar to that seen on freshly isolated basophils, CCR4 and CXCR2 expression was higher in BMBs by comparison (see Fig E2, C).

Sorted FcεRI⁺CD49b⁺c-kit⁻ basophils (see Fig E2, A) displayed chemotaxis toward gradients of CCL2, CXCL8, and CCL17, and CCL22 in a concentration-dependent manner (Fig 2, C-F). BMBs did not migrate in response to stem cell factor, which served as a negative control here because basophils do not express the stem cell factor receptor (c-kit) or in response to CXCL12, CXCL13, IL-3, or papain alone (Fig 2 and data not shown), suggesting a unique role for papain-induced chemokines in basophil recruitment to pLNs. Basophil migration in the presence of equivalent chemokine concentrations in the upper and lower chambers was similar to that observed in the absence of chemokine, indicating that chemokine gradients stimulated chemotaxis rather than chemokinesis (Fig 2, C-F). These results indicated that murine basophils express functional CCR2, CCR4, and CXCR2 receptors.

Role of CCR4-CCL17/CCL22 axis in papain-elicited basophil migration

CCR4 and its ligands, CCL17 and CCL22, have been linked to T_H2-mediated inflammation.¹⁵ To define the role of this axis in papain responses, we immunized WT or *Ccr4*^{-/-} mice with papain and quantified basophils in pLNs 3 days after immunization. In naive unimmunized WT and *Ccr4*^{-/-} mice, basophil frequencies were similar in the spleen and BM, and T-cell, B-cell, and dendritic cell (DC) frequencies in the pLNs were also comparable (see Fig E3 in this article's Online Repository at www.jacionline.org). In contrast, basophil accumulation in pLNs after papain immunization was reduced by 60% in mice lacking CCR4 compared with WT mice (Fig 3, A). This disparity most likely did not result

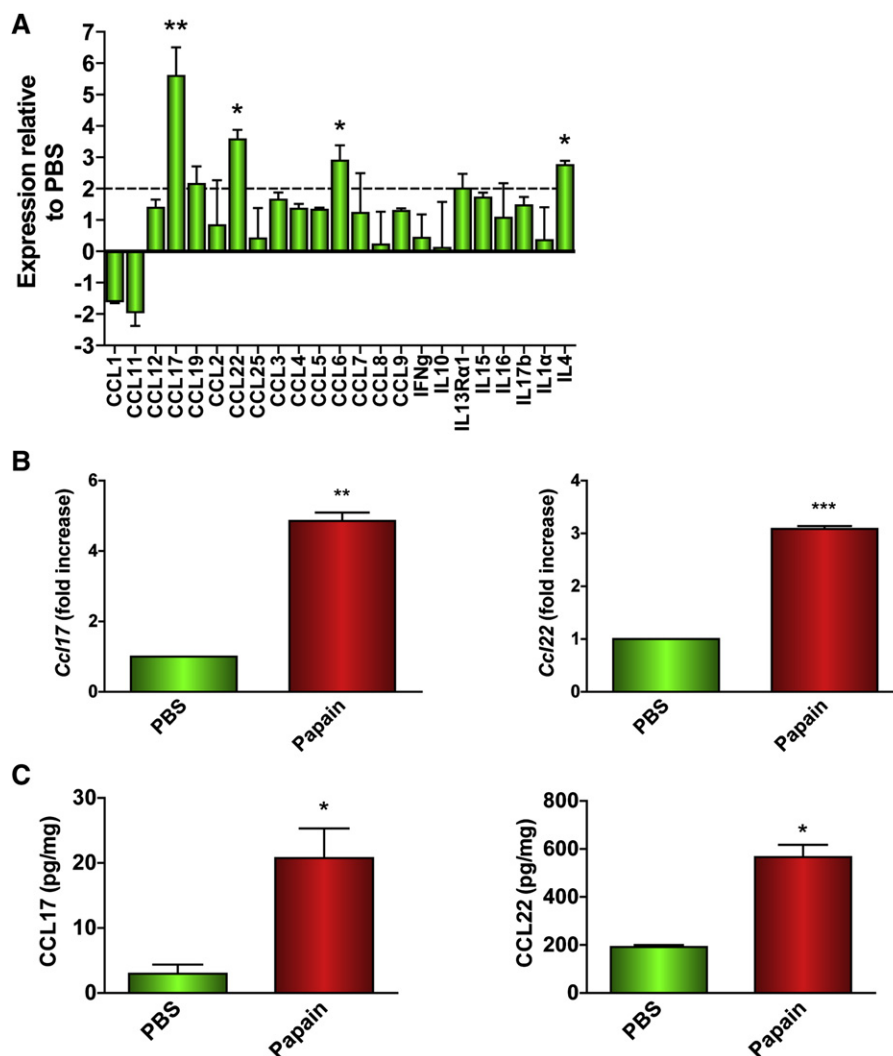


FIG 1. Inflammatory response of draining pLNs to footpad immunization with papain. **A**, qPCR array analysis of RNA from pLN RNA of PBS- or papain-treated mice. ** $P < .001$ and * $P < .05$, 2-way ANOVA. **B** and **C**, *Ccl17* and *Ccl22* mRNA (Fig 1, **B**) or CCL17 and CCL22 protein (Fig 1, **C**) expression in LNs 2 days after PBS or papain immunization was determined by using qPCR or ELISA, respectively. * $P < .03$, ** $P = .004$, and *** $P = .0007$, paired t test. Data represent 3 experiments with at least 3 mice per group in each.

from intrinsically defective pLN responses to papain because CCL17 and CCL22 concentrations were similar in pLNs from WT and *Ccr4*^{-/-} papain-treated mice (Fig 3, **C**). Papain-induced T_H2 differentiation was also reduced by CCR4 deficiency because there were 60% fewer IL-4⁺CD4⁺ T cells in the pLNs of *Ccr4*^{-/-} mice compared with WT mice 4 days after papain injection (Fig 3, **B**). Collectively, these results indicate a significant role of CCR4 in the immune response to papain.

Papain induces T-cell production of basophil-attracting chemokines independently of DCs

We determined the cellular source of CCL17 and CCL22 in pLNs after papain immunization by characterizing purified pLN cell populations (see Fig E4, **A**, in this article's Online Repository at www.jacionline.org). Papain increased *Ccl17* and *Ccl22* expression in LN T cells but not B cells (see Fig E4, **B**). *Ccl17* and *Ccl22* were significantly upregulated in papain-treated

CD4⁺ but not CD8⁺ T-cell populations compared with control cells (Fig 4, **A**). Although previous studies indicated that DCs and macrophages are potent sources of these chemokines,^{16,17} *Ccl17* and *Ccl22* expression was much higher in the pLN T-cell fraction than in the non-T-cell compartment, which can include B cells, macrophages, MCs, natural killer cells, and DCs (Fig 4, **A**). Accordingly, treatment of purified splenic macrophage/DC populations with papain *in vitro* did not induce significant *Ccl17/Ccl22* expression (see Fig E4, **C**).

Consistent with a published study,¹⁰ we also observed infiltration of migratory dermal DCs (CD8 α ⁻CD11c⁺CD205⁺)^{18,19} in pLNs 2 days after papain immunization (see Fig E5, **A**, in this article's Online Repository at www.jacionline.org). However, *Ccr4*^{-/-} mice had reduced basophil migration to pLNs in response to papain compared with that seen in WT mice despite equivalent numbers of pLN DCs (Fig 3, **A**, and see Fig E5, **A**). To formally evaluate the function of DCs in papain-induced chemokine responses, we used *Cd11c-Dtr-egfp* transgenic mice.

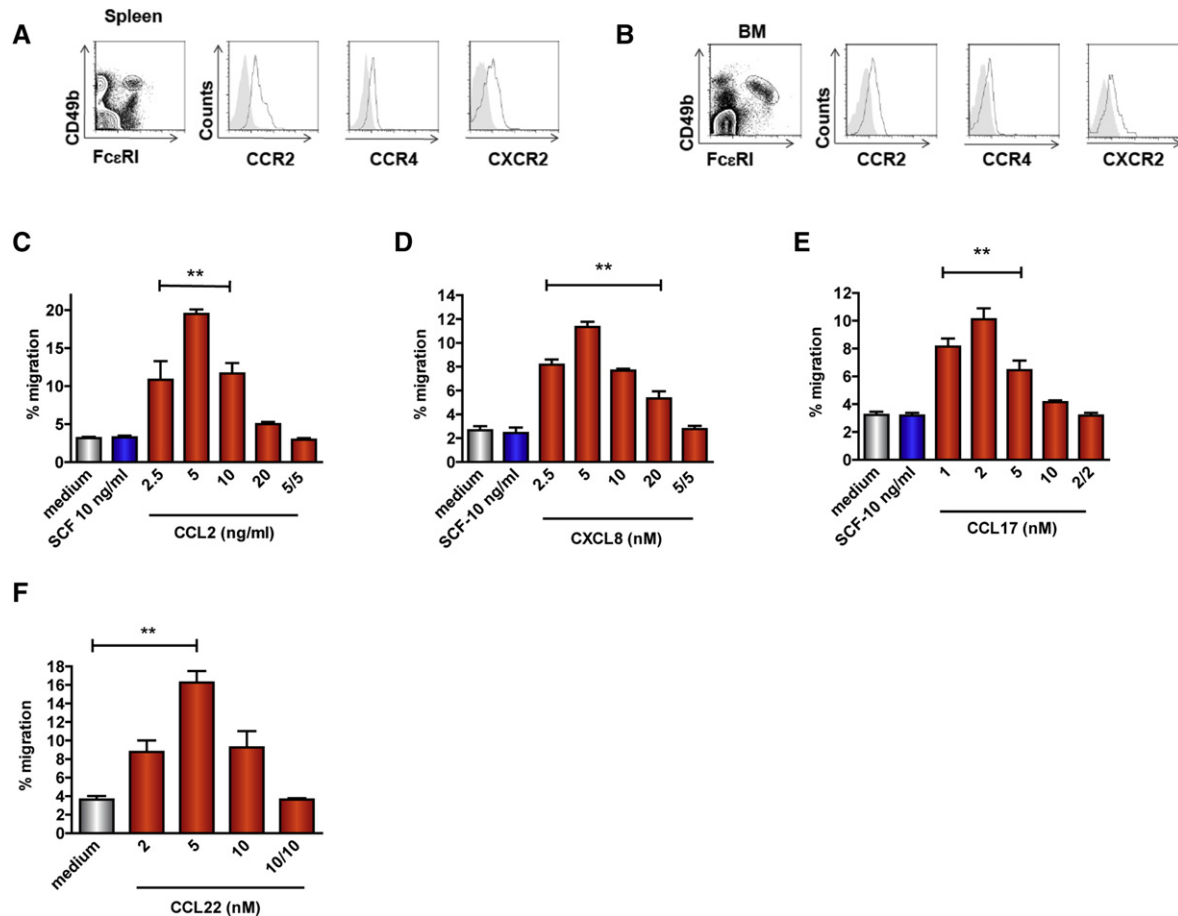


FIG 2. Basophil chemokine receptor expression and chemotaxis. **A** and **B**, Percentages of basophils in live, non-T non-B splenic cells (Fig 2, **A**) or freshly isolated total BM (Fig 2, **B**, left) and chemokine receptor expression (open histograms) in basophils (right); shaded histograms represent isotype controls. **C-F**, Chemotaxis of BMBs sorted from c-kit⁺ cells in response to CCL2 (Fig 2, **C**), CXCL8 (Fig 2, **D**), CCL17 (Fig 2, **E**), or CCL22 (Fig 2, **F**). Data are means $\pm$ SEMs of 2 to 3 experiments with BMBs from 2 to 3 mice per experiment. SCF, Stem cell factor. ** $P < .001$, indicated chemokine concentration versus control, 1-way ANOVA.

Consistent with previous studies,¹¹ when injected with diphtheria toxin 1 day before papain immunization and then treated with papain and examined 2 days later, the pLNs of these mice were largely devoid of DCs (MHC^{hi}CD11c⁺; see Fig E5, **B**). DC deficiency did not reduce either papain-induced basophil accumulation in pLNs (Fig 4, **B**, and see Fig E5, **C**) or upregulation of *Ccl17* and *Ccl22* expression (see Fig E5, **D**). Collectively, these results indicated that DCs are neither sufficient nor required for chemokine-mediated recruitment of basophils to pLNs by papain.

To ascertain whether T cells required prior APC-mediated priming to respond to papain, we measured chemokine expression in naive T cells purified from unimmunized mice that were pulsed with papain *in vitro* for 20 hours (see Fig E6, **A**, in this article's Online Repository at www.jacionline.org). Papain induced *Ccl17* and *Ccl22* mRNA expression (Fig 4, **C**, and see Fig E6, **B**) and CCL22 protein expression (Fig 4, **D**) in unprimed naive T cells. We detected CCL17 protein in supernatants from cells stimulated with IL-4 *in vitro*, which has been reported to induce strong *Ccl17* expression in human CD4⁺ T cells through signal transducer and activator of transcription (STAT) 6 binding sites in the *Ccl17* promoter.²⁰ We attribute our inability to detect CCL17 after papain treatment to the fact that absolute CCL17

amounts were quite lower than CCL22 amounts in culture supernatants, most likely less than assay detection limits. The proinflammatory effect of papain was dependent on its protease activity because heat inactivation of papain abolished chemokine production by T cells (Fig 4, **C** and **D**).

Papain activates naive T cells through protease-activated receptor 2-dependent mechanisms

The finding that papain stimulated antigen-inexperienced T cells to produce CCL17 and CCL22 through its protease activity provoked the hypothesis that T cells sense papain through a protease-activated receptor (PAR). Four PARs (PAR1-PAR4) have been identified that are activated uniquely by proteolytic cleavage at sequences within their amino termini to expose a tethered ligand.²¹ Because previous studies have indicated a role for PAR2 in T_H2-mediated inflammation through unclear mechanisms,²² we evaluated its importance for papain-evoked T-cell immunity. We detected PAR2 on 10% to 15% of human peripheral blood naive T cells (Fig 5, **A**). Naive and effector/memory splenic T cells from WT but not *Par2*^{-/-} mice also expressed PAR2 to varying degrees (see Fig E7, **A** and **B**, in this article's Online

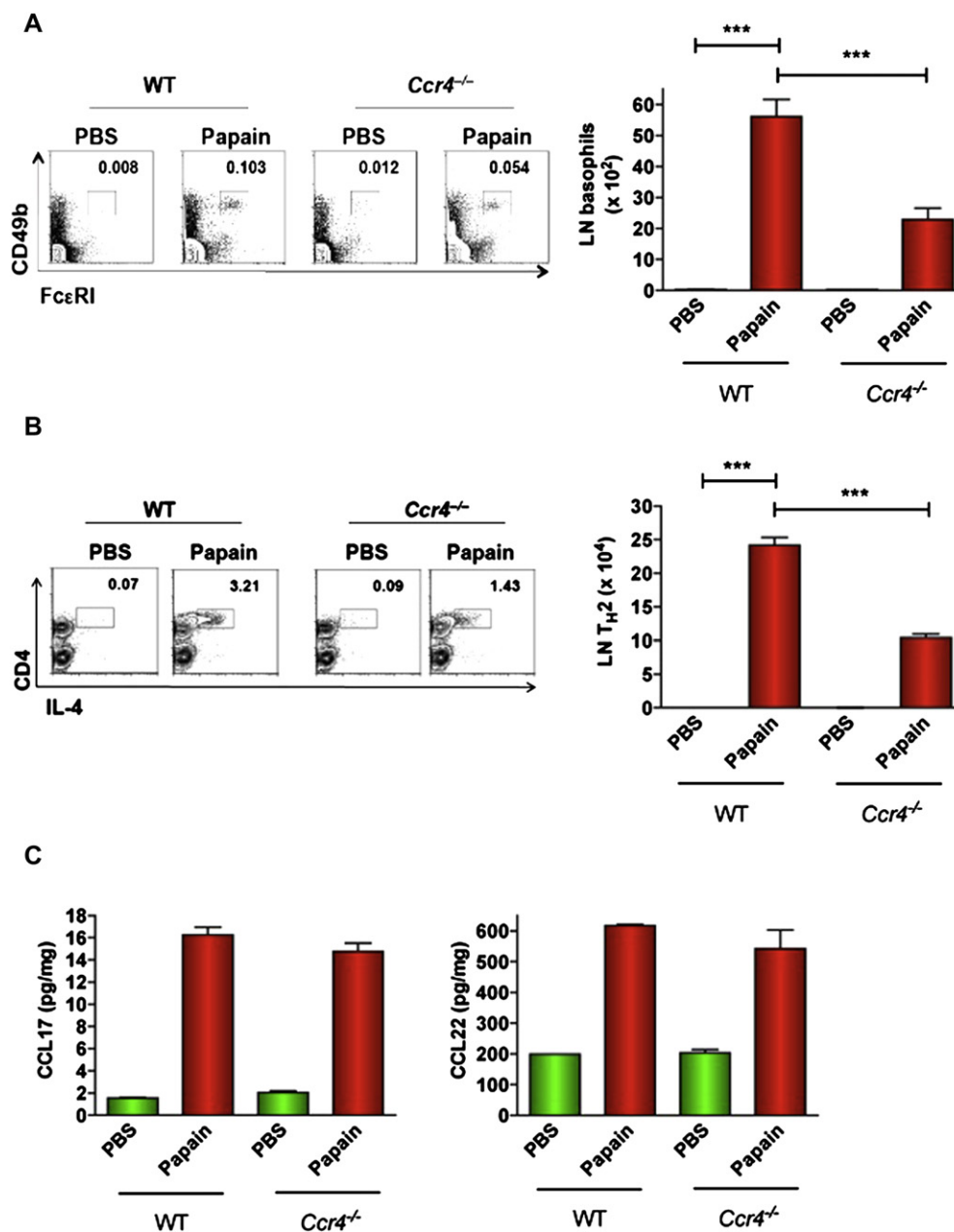


FIG 3. Role of CCR4 in papain-induced basophil trafficking to pLNs. **A** and **B**, WT or *Ccr4*^{-/-} mice were immunized with PBS or papain, followed by enumeration of pLN basophils after 3 days (Fig 3, A) or IL-4⁺ CD4⁺ T cells (after 6 hours of restimulation with phorbol 12-myristate 13-acetate/ionomycin) after 4 days (Fig 3, B). Numbers above outlined areas are percentages of total cells from a representative experiment. Graphs show means $\pm$ SEMs of 3 experiments. ****P* < .001, 1-way ANOVA. **C**, Chemokine concentrations in pLNs 2 days after footpad immunization with papain were determined by means of ELISA (2 experiments with 3 mice per group).

Repository at www.jacionline.org). Papain-evoked basophil recruitment to pLNs (Fig 5, B) and *Ccl17* and *Ccl22* expression (Fig 5, C) were severely diminished in *Par2*^{-/-} compared with WT mice, although chemotaxis of purified PAR2-deficient basophils toward a CCL22 gradient *in vitro* was intact (see Fig E7, C). Papain elicited much less *Ccl17* and *Ccl22* expression in purified CD4⁺ T cells from *Par2*^{-/-} mice compared with that seen in WT mice (see Fig E7, D), and the small-molecule PAR2 antagonist

ENMD-1068 significantly reduced papain-induced chemokine expression in human CD4⁺ T cells (Fig 5, D). Inhibitors of p38 and Janus kinase (Jak) 3 activity, but not a c-Jun N-terminal kinase inhibitor, also strongly reduced papain-evoked *Ccl17* and *Ccl22* gene expression in both murine (see Fig E7, E) and human (Fig 5, E) CD4⁺ T cells. Collectively, these results suggest that papain-elicited CCL17 and CCL22 production by T cells depends on PAR2, p38, and Jak3.

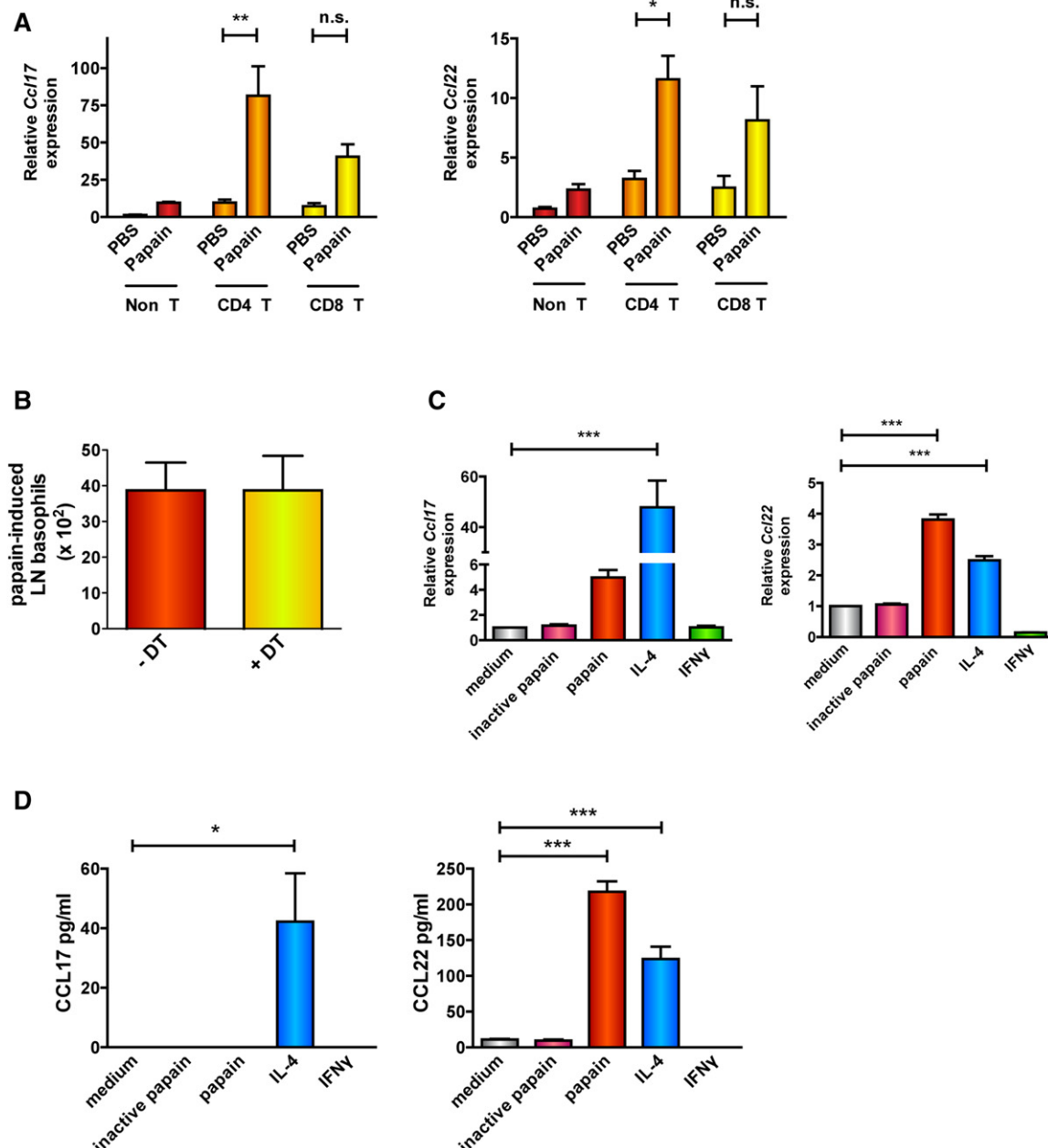


FIG 4. Papain induces *Ccl17* and *Ccl22* expression in naive T cells independently of DCs. **A**, Chemokine expression in CD4, CD8, or non-T fractions purified from the pLNs of PBS- or papain-immunized mice. *n.s.*, Not significant. * $P < .05$ and ** $P < .005$, 1-way ANOVA. **B**, *Cd11c-Dtr* transgenic mice were injected with PBS or diphtheria toxin (DT) 1 day before immunization with papain. Basophil numbers in the pLNs were quantified 2 days after papain treatment. **C** and **D**, Chemokine gene expression (Fig 4, C) or protein secretion (Fig 4, D) was determined in CD4⁺ T cells sorted from spleens of unimmunized mice and cultured *in vitro* with papain for 20 hours (Fig 4, C) or 3 days (Fig 4, D), respectively. Data represent 2 to 3 experiments with 3 mice per group. * $P < .05$ and *** $P < .0005$, 1-way ANOVA.

Requirement of T cells for the immune response to papain *in vivo*

Because CD4⁺ T cells appeared to be a major source of CCL17 and CCL22 immediately after papain injection, we evaluated their role in basophil migration to pLNs using *Cd4*^{-/-} mice (see Fig E8, A, in this article's Online Repository at www.jacionline.org). Naive WT and *Cd4*^{-/-} mice had similar percentages of basophils in spleens and BM (see Fig E8, B), indicating that CD4⁺ T

cells are not required for basophil differentiation and survival in the absence of immune challenge. However, basophils did not appear in pLNs of CD4-deficient mice after papain immunization (Fig 6, A), despite equivalent numbers of total pLN DCs and influx of migratory dermal DCs with papain treatment (see Fig E8, C and D), and allergen-induced upregulation of pLN *Ccl17* and *Ccl22* was absent in these mice (Fig 6, B). We also failed to detect basophils and chemokine upregulation in pLNs of

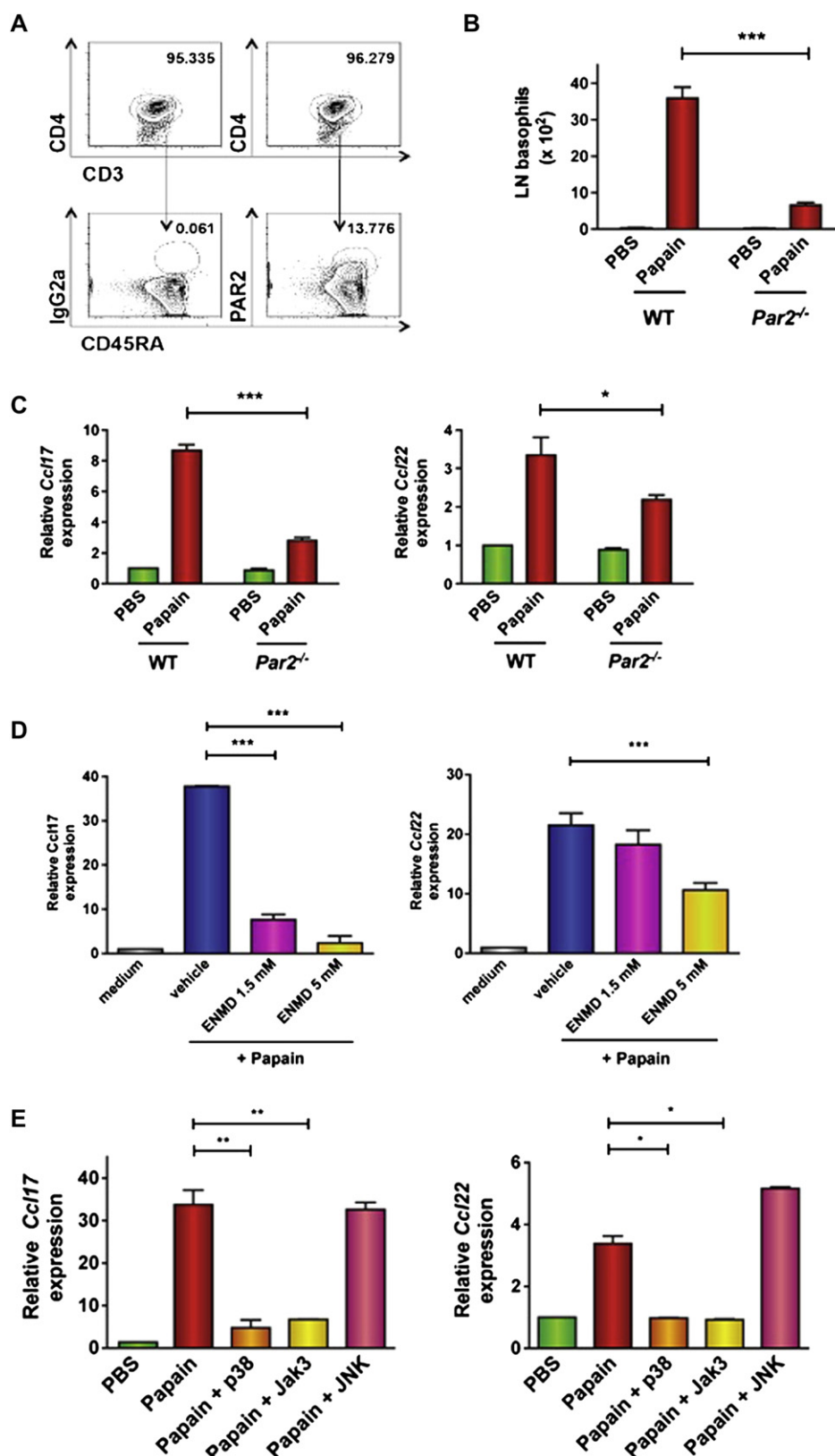


FIG 5. Papain induces chemokine production and basophil trafficking through PAR2-dependent mechanisms. **A**, PAR2 expression in human peripheral blood naive CD4 T cells (CD45RA⁺). Numbers above outlined areas represent percentages of total live cells. **B** and **C**, C57/BL6 WT or *Par2*^{-/-} mice were immunized in the footpad with papain, followed by enumeration of basophils (Fig 5, **B**) or *Ccl17* and *Ccl22* gene expression (Fig 5, **C**) in the pLNs. Data represent 3 experiments with 3 mice per group.

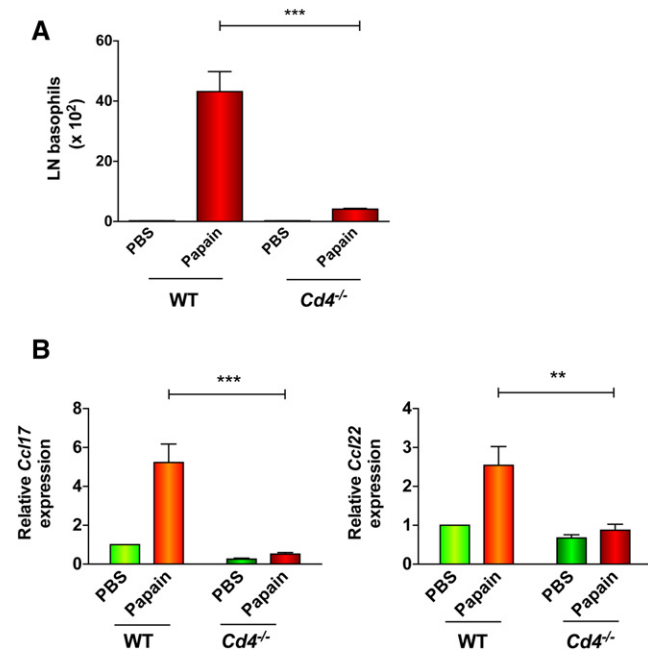


FIG 6. Requirement of T cells for basophil migration to LNs and production of chemokines in response to papain. Basophil numbers (**A**) or *Ccl17* and *Ccl22* gene expression (**B**) in the pLNs of PBS- or papain-treated WT or *Cd4*^{-/-} mice. ***P* = .003 and ****P* < .0001, 1-way ANOVA. Data represent 3 experiments with at least 3 mice per group.

Tcrα^{-/-} mice, which lack αβ T cells (see Fig E9 in this article's Online Repository at www.jacionline.org). To determine whether the presence of CD4⁺ T cells could restore papain-induced basophil migration to pLNs in *Cd4*^{-/-} mice, we transferred 12.5 million CD4⁺ T cells into these mice either through intravenous or direct footpad injection. However, we detected a thousand-fold fewer T cells in pLNs of the reconstituted *Cd4*^{-/-} mice than were present in WT pLNs independent of papain immunization (see Fig E10, A, in this article's Online Repository at www.jacionline.org). Increasing the number of donor CD4⁺ T cells did not improve engraftment (data not shown). This limitation precluded meaningful analysis of basophil numbers in pLNs of papain-treated recipients (see Fig E10, B).

Papain induces PAR2-dependent IL-4 production by naive T cells and basophils

Jak3-mediated phosphorylation of STAT5 and STAT6 is integral for *Il4* transcription in T cells.²³ Because a Jak3 inhibitor strongly reduced CCL17 and CCL22 production by naive T cells (Fig 5, E), we hypothesized that papain also induces *Il4* expression. Purified naive CD4⁺ T cells from unimmunized mice upregulated *Il4* mRNA within 3 hours of *in vitro* stimulation with papain (Fig 7, A). Naive CD4⁺ T cells from unimmunized DO11.10/4get/*Rag1*^{-/-} mice, which should not mount a robust antigen-restricted response to papain, also had increased green fluorescent protein intensity after papain treatment *in vitro*,

indicating IL-4 production (Fig 7, B). This result provoked the hypothesis that papain could induce an initial burst of IL-4 secretion by naive T cells that might act in an autocrine/paracrine manner to upregulate chemokine expression and recruit basophils. To evaluate the role of IL-4 in papain-evoked T-cell responses, we stimulated CD4⁺ T cells from *Il4*^{-/-} mice with papain. Such cells had increased *Ccl17/Ccl22* expression after papain treatment that was comparable with that seen in WT cells (see Fig E11 in this article's Online Repository at www.jacionline.org). Thus IL-4 itself is not required for papain-induced chemokine expression in T cells.

Finally, because the receptor mediating papain-induced *Il4* expression in basophils¹⁰ was unknown, we determined whether PAR2 was required for basophil responses to papain. BMBs expressed abundant surface PAR2 (Fig 7, C), and *Par2* gene deletion nearly abolished papain-evoked *Il4* expression in BMBs (Fig 7, D). These results suggest that PAR2 is the principal papain receptor on T cells and basophils and that PAR2 mediates an early cytokine/chemokine loop that drives T_H2 immune responses to papain in mice.

DISCUSSION

We have elucidated an innate allergen-recognition pathway in mice mediated by conventional naive T cells and requiring PAR2, which not only induces basophil recruitment to pLNs through chemokine production but also provides an immediate source of IL-4 before induction of the antigen-specific T_H2 immune response. The function or functions of PAR2 in T cells were previously unknown. Although prior work demonstrated that *Par2*^{-/-} mice had reduced OVA-induced T-cell differentiation and cytokine production relative to WT mice, PAR2-activating peptide alone did not stimulate cytokine production by WT murine CD4⁺ T cells.²² We show here that both murine and human naive T cells express surface PAR2 and respond directly to papain in a PAR2-, p38 mitogen-activated protein kinase-, and Jak3 kinase-dependent manner, and PAR2 appears to be crucial for the immune response to papain *in vivo*.

Published work has also failed to establish the primary cellular receptor for papain or other protease allergens in immune cells. Human eosinophil degranulation in response to papain required the protease activity of papain but not PAR2.²⁴ By contrast, Kouzaki et al²⁵ reported that papain-evoked cytokine production by epithelial cells was partially reduced by *Par2* small interfering RNA compared with control values, although the effectiveness of small interfering RNA to reduce PAR2 expression was not demonstrated in this study. Because we detected residual chemokine production to papain in the pLNs of *Par2*^{-/-} mice, not all responses to papain might involve PAR2. Given the promiscuity of other proteases (eg, thrombin) for multiple PARs (PAR1, PAR3, and PAR4) and the ability of papain to induce alterations in membrane charge²⁶ or proteolytically cleave other surface receptors on T cells (eg, CD25),²⁷ papain might also induce T-cell activation, basophil activation, or both through other PARs or through PAR-independent mechanisms. Alternate cellular sources of

P* < .05 and **P* < .0001, 1-way ANOVA. **D** and **E**, Chemokine expression in human peripheral blood CD4⁺ T cells cultured for 20 hours with medium or papain after pretreatment for 1 hour with vehicle (dimethyl sulfoxide) alone, the PAR2 antagonist ENMD-1068 (Fig 5, D), or the inhibitors of p38, JAK3, or c-Jun N-terminal kinases (Fig 5, E). Data are means ± SEMs of 3 experiments. **P* < .05 and ***P* < .005, 1-way ANOVA.

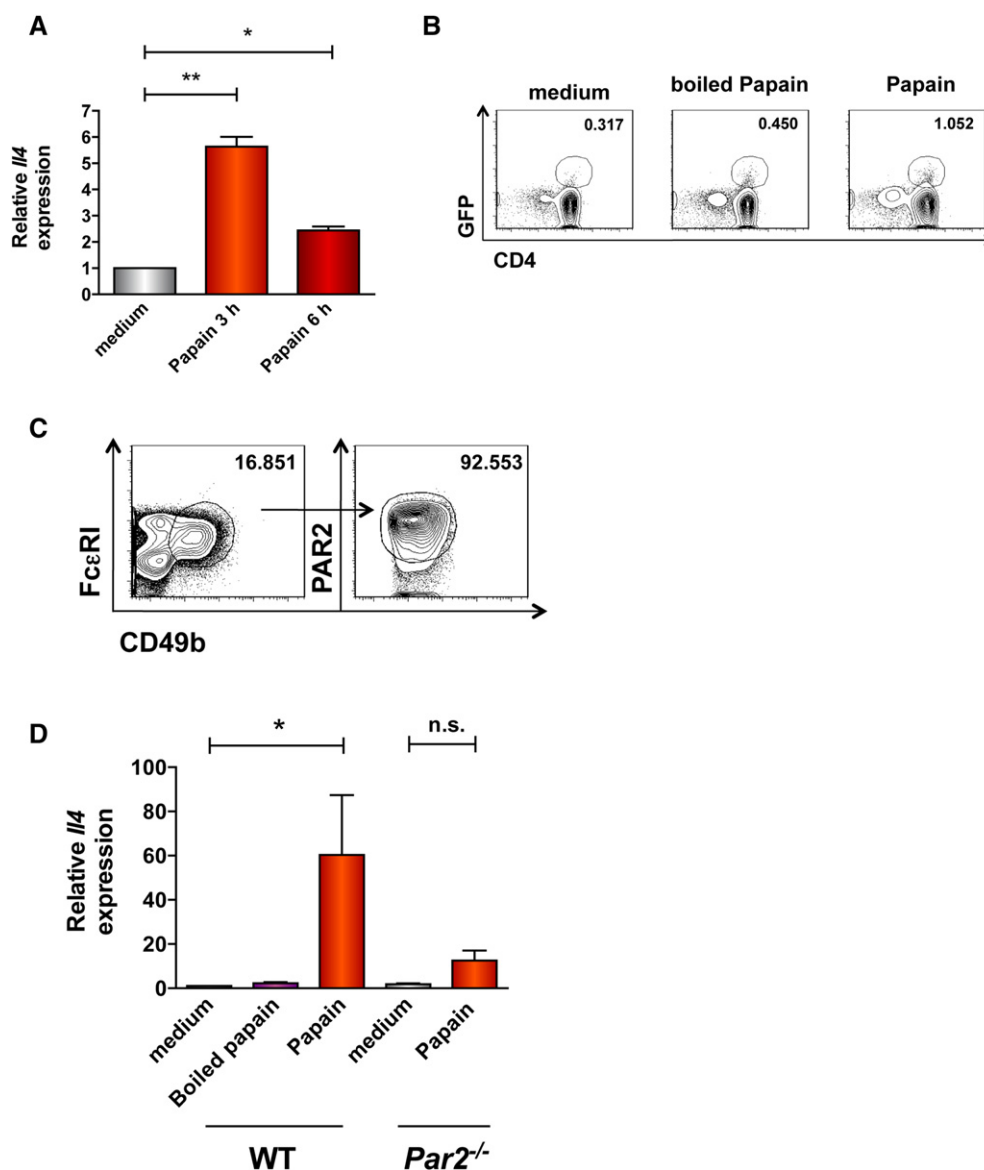


FIG 7. Papain elicits IL-4 expression in basophils and T cells through PAR2-dependent mechanisms. **A**, IL4 expression in naive T cells from unimmunized C57Bl/6 mice stimulated with papain was determined by using qPCR. $*P < .05$ and $**P = .002$, 1-way ANOVA. **B**, IL-4 expression (green fluorescent protein [GFP]) in papain-treated splenocytes from DO11.10/4get/*Rag1*^{-/-} mice. Data are from a single experiment with 2 mice per group representative of 2 similar experiments. **C**, PAR2 on BMBs was determined by means of flow cytometry. Numbers above outlined areas indicate percentages of total live cells. **D**, IL4 gene expression in BMBs from C57Bl/6 or *Par2*^{-/-} mice was determined by means of qPCR. Data represent 2 to 3 experiments with 3 mice per experiment. n.s., Not significant. $*P < .05$, 1-way ANOVA.

chemokines could also compensate for the reduced pLN response to papain in the absence of PAR2 *in vivo*.

In the present study we demonstrated that T cells were required for CCL17 and CCL22 production and basophil accumulation in pLNs after papain injection. Other leukocytes that have been previously reported to be reservoirs of these chemokines, including MCs, macrophages, and DCs, apparently made minor contributions to papain-elicited immunity. These results are consistent with previous work showing that basophil migration to tissues infected with *Nippostrongylus brasiliensis* is absent in *Rag2*^{-/-} mice.^{28,29} In contrast, a separate study reported only a modest decrease in basophil recruitment to draining LNs of T cell-depleted mice (using anti-CD3 antibody) injected with papain plus OVA

relative to control values.⁹ These results suggest that an immune response to papain is distinct from that induced by simultaneous exposure to inert and protease allergens. Generation of mice with conditional deletion of PAR2 in T cells or chimeras of T cell-deficient mice containing WT or PAR2-deficient BM might further define the functions of PAR2-expressing T cells in T_H2 immunity.

In agreement with Sokol et al¹¹ and Nakagawa et al,³⁰ we found that decreased pLN basophil numbers in papain-immunized *Ccr4*^{-/-} mice relative to WT mice led to reduced frequencies of IL-4⁺ CD4 T cells in these same LNs. Because naive T cells do not express CCR4, these results suggest a nonredundant role of basophils in the antigen-restricted T_H2 response to papain. Our work also suggests that quantification of total IL-4⁺ T-cell

numbers (as has been reported in some studies) would not distinguish differentiated, antigen-specific T_H2 cells primed to produce IL-4 from naive $CD4^+$ T cells stimulated through PAR2 in the absence of APC-mediated priming. Thus the contribution of basophils to T_H2 immunity overall is still unclear.

The relevance of this pathway to allergy pathogenesis in human subjects requires further study. We show that the protease allergen papain has the capacity to stimulate human and murine naive T cells and murine basophils through PAR2-dependent mechanisms. It is unclear whether human basophils express PAR2 or become activated by papain.^{31,32} It is also unknown whether human MCs respond to papain, although MCs are dispensable for immunity to papain in mice.^{6,10} Accordingly, we did not detect PAR2 expression on murine BM-derived mast cells (data not shown), nor did they upregulate *Il4* expression in response to papain (see Fig E12 in this article's Online Repository at www.jacionline.org).

In a previous study naive peripheral blood T cells from allergic asthmatic patients produced CCL17, whereas those from nonallergic control subjects did not.³³ A recent population study of Korean children demonstrated significant correlation of atopy, serum IgE levels, and eosinophilia with a single nucleotide polymorphism in *Par2*, which increased PAR2 expression in PBMCs because of enhanced mRNA stability.³⁴ Our studies provide a molecular mechanism linking these observations, namely that allergens could induce pathogenic T-cell responses in atopic subjects as a result of increased PAR2 expression. Further elucidation of the mechanisms underlying allergen-elicited direct programming of naive T cells (ie, MHC independent) could lead to the identification of new targets for the treatment of allergies and helminth infection.

Key messages

- Naive T cells respond to the allergen papain through a G protein-coupled receptor, PAR2, to produce IL-4 and chemokines.
- Basophils are recruited to LNs by papain through chemokine- and PAR2-dependent mechanisms and respond with a burst of IL-4 production.
- PAR2 antagonists might subvert T_H2 immunity by reducing the inflammatory response to allergens, such as papain.

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METHODS

Immunization

Mice were immunized subcutaneously in the rear footpad with papain (50 mL of 1 μ g/mL solution in PBS) or PBS alone, as previously described.^{E1,E2} For DC depletion, *Cd11c-Dtr* mice were depleted of DCs with a single intraperitoneal injection of diphtheria toxin (100 ng in 100 mL of PBS) 1 day before papain immunization.

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-anti-mouse Fc ϵ RI (MAR-1), allophycocyanin-anti-mouse Fc ϵ RI (MAR-1), phycoerythrin (PE)-anti-CD49b (DX5), FITC-anti-c-kit (2B8), allophycocyanin-anti-c-kit (2B8), allophycocyanin-anti-CD11c (N418), PE-anti-CD11c (N418), FITC-anti-CD11c (N418), FITC-anti-CD3 α (145-2c11), allophycocyanin-eFluo780-anti-CD3 α (17A2), FITC-anti-CD4 (GK1.5), PE-anti-CD4 (GK1.5), allophycocyanin-anti-CD8 β , PE-Cy5-anti-CD8 β , peridinin-chlorophyll-protein complex-Cy5.5-anti-CD25 (PC61.5), PE-Cy7-anti-CD44 (1M7), allophycocyanin-anti-CD205 (205yekta), allophycocyanin-anti-CD62 ligand (MEL-14), PE-anti-IL-4 (11B11), and PE-anti-MHCII (M5/114.15.2), and anti-human CD45RA were purchased from eBioscience (San Diego, Calif). Anti-human CD4, PE-anti-CD19 (1D3), FITC-anti-CD8 α , allophycocyanin-anti-CCR3 (83103), PE-anti-CCR5 (C34-3448), allophycocyanin-anti-CCR6 (140706), anti-CXCR4 (2B11), PE-anti-CXCR5 (2G8), and Fc Block (2.4G2) were from BD Biosciences (Franklin Lakes, NJ). FITC-anti-human PAR2 (SAM-11), anti-mouse PAR2 (sc08207), and allophycocyanin-anti-CCR1 (catalog no. sc-6125) were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). PE-anti-CCR4 (2G12), PE-anti-CCR7 (4B12), and allophycocyanin-anti-CXCR2 (TG11) were from BioLegend (San Diego, Calif); anti-CCR2 was from Capralogics (Hardwick, Mass). Q-Dot 605-anti-human CD3 was from Invitrogen. Murine anti-PAR2 was conjugated to Alexa Fluor 594 by using the Monoclonal Antibody Labeling Kit (Molecular Probes, Eugene, Ore). Papain (from *Carica papaya*, catalog no. 5125, lot no. D00073805) was from EMD Biosciences (San Diego, Calif), and diphtheria toxin was obtained from Sigma-Aldrich (St Louis, Mo).

The PAR2 small-molecule antagonist ENMD-1068 (Enzo Life Sciences, Farmingdale, NY) was dissolved in PBS and used at final concentrations of 1.5 and 5 mmol/L, as indicated. Cells were pretreated for 1 hour before papain stimulation. Diphtheria toxin (Sigma-Aldrich, 100 mg in 100 μ L PBS) was injected intraperitoneally 24 hours before papain immunization.

Flow cytometry

For detection of basophils and chemokine receptors in naive mice, BM cells and splenocytes were depleted of CD90.2⁺ T cells and B220⁺ cells by using magnetic bead sorting (Miltenyi Biotec). All cells were incubated with murine Fc Block (2.4G2) and then stained with primary antibodies. Fluorescence was measured with a FACSCalibur (BD Biosciences) or LSRII (BD Biosciences) flow cytometer and analyzed with Flowjo software (Tree Star, Inc, Ashland, Ore).

Adoptive T-cell transfers

Spleens were isolated from age- and sex-matched WT C57Bl/6 or *Par2*^{-/-} donors. After red cell lysis, CD4⁺ T cells were isolated from single-cell suspensions by means of magnetic bead sorting with the CD4⁺ T cell isolation kit II, according to the manufacturer's instructions (Miltenyi Biotec). CD4⁺ cells (12.5×10^6) were resuspended in sterile PBS (200 μ L) and injected either intravenously into the tail vein or subcutaneously into the rear footpads of age- and sex-matched B6 *Cd4*^{-/-} recipient mice. Two days after each transfer, the recipients were either left untreated or injected subcutaneously with 50 μ L of papain (1 mg/mL) or PBS into the rear footpad. Three days later, recipient mice were evaluated for engraftment and papain responses by means of quantification of CD4⁺ T cells and basophils in spleens and pLNs by using flow cytometry.

Measurement of papain-specific IgE

Antigen-specific IgE levels were measured in serum of human serum albumin- or papain-immunized mice (5 mice per group), as described previously.^{E3}

LN cell purifications and *in vitro* stimulations

B cells, total T cells, or CD4⁺ T cells were purified from pLNs by using B220, CD90.2, or CD4 microbeads, respectively (Miltenyi Biotec). RNA was prepared with the RNeasy kit (Qiagen). CD8⁺ T cells were purified from LN cell preparations depleted of CD4⁺ cells by using CD90.2 microbeads. For *in vitro* stimulation, naive CD4⁺ T cells were purified from the spleen by means of negative selection with the naive T cell isolation kit II (Miltenyi Biotec), followed by selection of CD62L⁺CD11c⁻ cells by means of flow cytometry. BM-derived mast cells were obtained from 4- to 6-week-old BM cultures grown in RPMI containing 10% FCS and IL-3 (30 ng/mL) as above. For macrophage isolation, tissue-culture plates were coated with 2 mL of 100% heat-inactivated FCS (Life Technologies) overnight at 4°C, as previously described.^{E4} Dishes were washed with cold PBS, followed by seeding of splenic cell suspensions (10^6 cells/mL). After 4 hours of incubation at 37°C, we decanted nonadherent cells by means of pipetting and washing with RPMI. Adherent cells were agitated in a solution of 0.2% EDTA and 5% FCS for 2 hours at 4°C. Cells were then seeded in RPMI containing 10% FCS (10^6 cells/mL) in 12-well plates and stimulated with PBS or papain for 20 hours. Human PBMCs were separated from leukapheresis samples obtained from the National Institutes of Health blood bank. Naive CD4⁺ T cells were isolated by means of magnetic bead sorting with the human naive CD4 T cell isolation kit (Miltenyi Biotec). cDNA was generated from 500 ng to 1 μ g of total RNA by using the SuperScript III Reverse Transcriptase Synthesis System (Invitrogen). *Ccl17* and *Ccl22* expression was determined by using *Ccl17* primer (Applied Biosystems primer ID Mm00516136_m1) and *Ccl22* (Applied Biosystems primer ID Mm00436439_m1) sets with a Taqman RT-PCR master mix (Applied Biosystems). Target gene values were normalized to β -actin values (primer ID Mm01205647_g1). Human chemokine expression was determined by using *Ccl17* (Applied Biosystems, primer ID Hs00171074_m1) and *Ccl22* (Applied Biosystems, primer ID Hs0017080_m1) primers. Target gene values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) values (Applied Biosystems, primer ID#Hs99999905_m1).

Real-time qPCR and RNA array

Cytokine and chemokine expression in whole pLN RNA was analyzed by using the RT² Profiler Mouse Inflammatory Cytokines and Receptors PCR array (catalog no. PAMM-011A; SA Biosciences, Qiagen, Valencia, Calif), according to the manufacturer's instructions.

ELISA

Homogenates were prepared from pLNs, as described elsewhere.^{E5} Naive CD4⁺ T cells purified from spleens were cultured in RPMI medium supplemented with 10% FBS for 3 days, followed by supernatant collection. The protein or supernatant samples were analyzed by using Quantikine ELISA kits (R&D Systems, Minneapolis, Minn) per the manufacturer's instructions.

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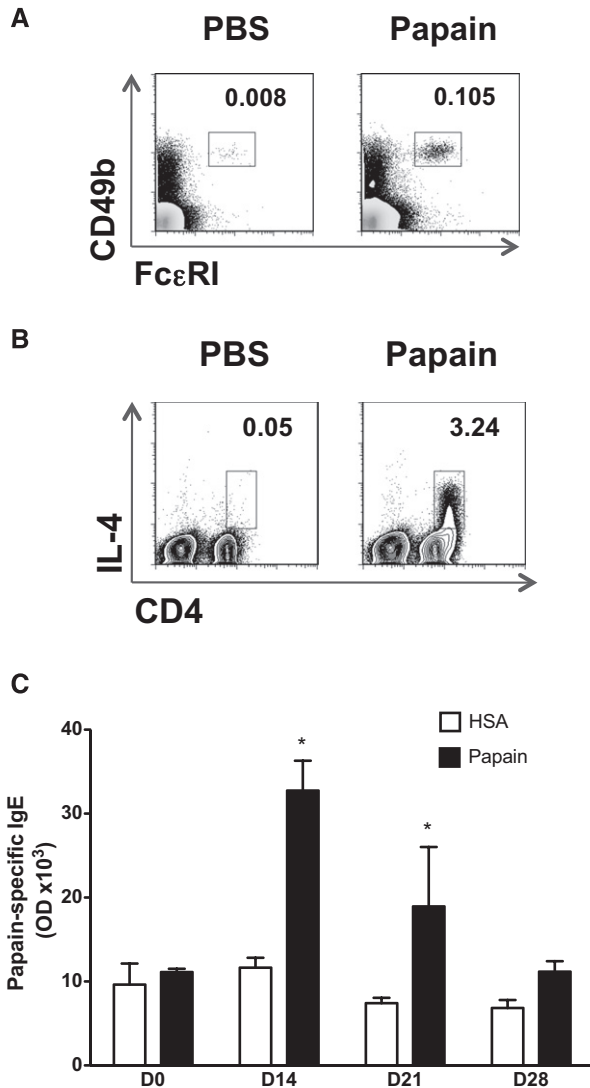


FIG E1. Papain induces a T_H2 immune response. **A** and **B**, Basophils ($Fc\epsilon RI^+CD49b^+$) in total live $c-kit^-$ population (Fig E1, **A**) or LN T_H2 cell ($CD4^+IL-4^+$) numbers (Fig E1, **B**) from pLNs of PBS- or papain-immunized mice were enumerated by means of flow cytometry 3 or 4 days after papain immunization, respectively. T cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL) plus ionomycin (1 mmol/L) for 6 hours *in vitro* before evaluation. Numbers above outlined areas represent percentages of live cells. **C**, Serum papain-specific IgE levels in human serum albumin (HSA)- or papain-immunized mice were determined by using ELISA. * $P = .02$, 2-way ANOVA.

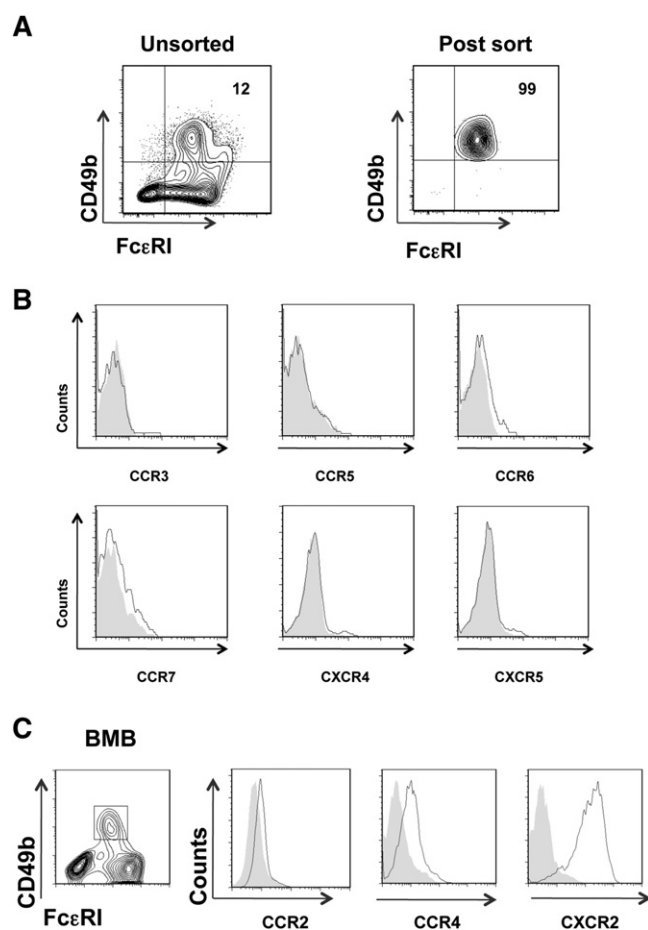


FIG E2. Chemokine receptor expression in murine BMBs. **A**, BMBs were sorted from BM cultures grown in IL-3 for 7 to 10 days, as described in the Methods section. Numbers indicate percentages of total live c-kit⁺ cells. **B** and **C**, BMBs were stained with the indicated chemokine receptor antibodies (*open histograms*); *shaded histograms* represent isotype control. Data are from a single experiment (1 mouse) representative of at least 3 independent experiments with 1 mouse per experiment.

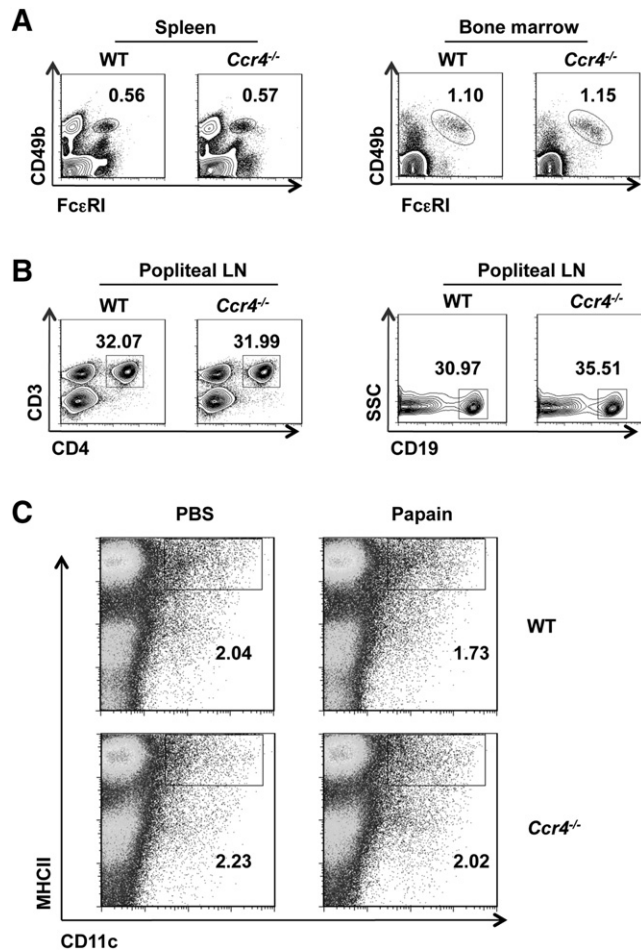


FIG E3. pLN leukocyte populations in naive WT and CCR4-deficient mice. **A** and **B**, Splenic and BM basophils (Fig E3, **A**) or pLN T (CD3⁺) and B (CD19⁺) lymphocytes (Fig E3, **B**) from naive C57Bl/6 WT or CCR4-deficient mice. **C**, pLN DCs (MHCII^{hi}CD11c⁺) from naive WT or CCR4-deficient mice. Numbers above outlined areas indicate percentage of total live cells. Data are representative of 2 to 3 independent experiments with 3 mice per experiment.

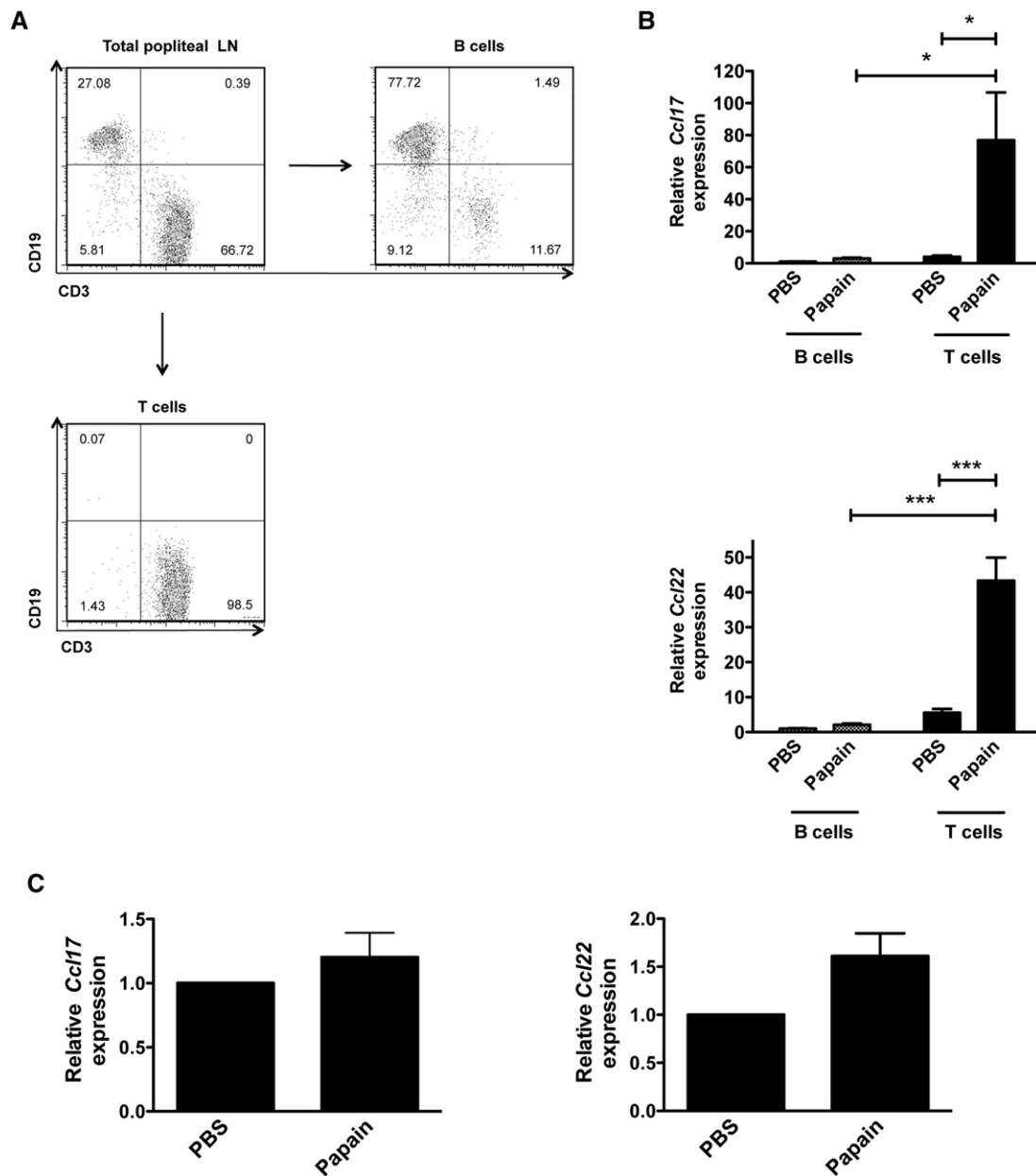


FIG E4. Characterization of chemokine expression in pLN cell populations. **A**, Purity of pLN lymphocyte populations isolated by means of magnetic bead sorting was determined by using flow cytometry with the indicated antibodies. Numbers in each quadrant are percentages of total live cells. **B**, *Ccl17* and *Ccl22* mRNA expression in B and T cells isolated from pLNs of papain-immunized mice was determined by using real-time PCR. * $P = .02$ and *** $P = .0001$, 1-way ANOVA. **C**, Splenic macrophages were treated with papain (100 $\mu\text{g}/\text{mL}$) for 20 hours, followed by quantification of *Ccl17* and *Ccl22* gene expression by using real-time PCR. Graphs represent means $\pm$ SEMs of 2 to 3 independent experiments with 2 to 3 mice.

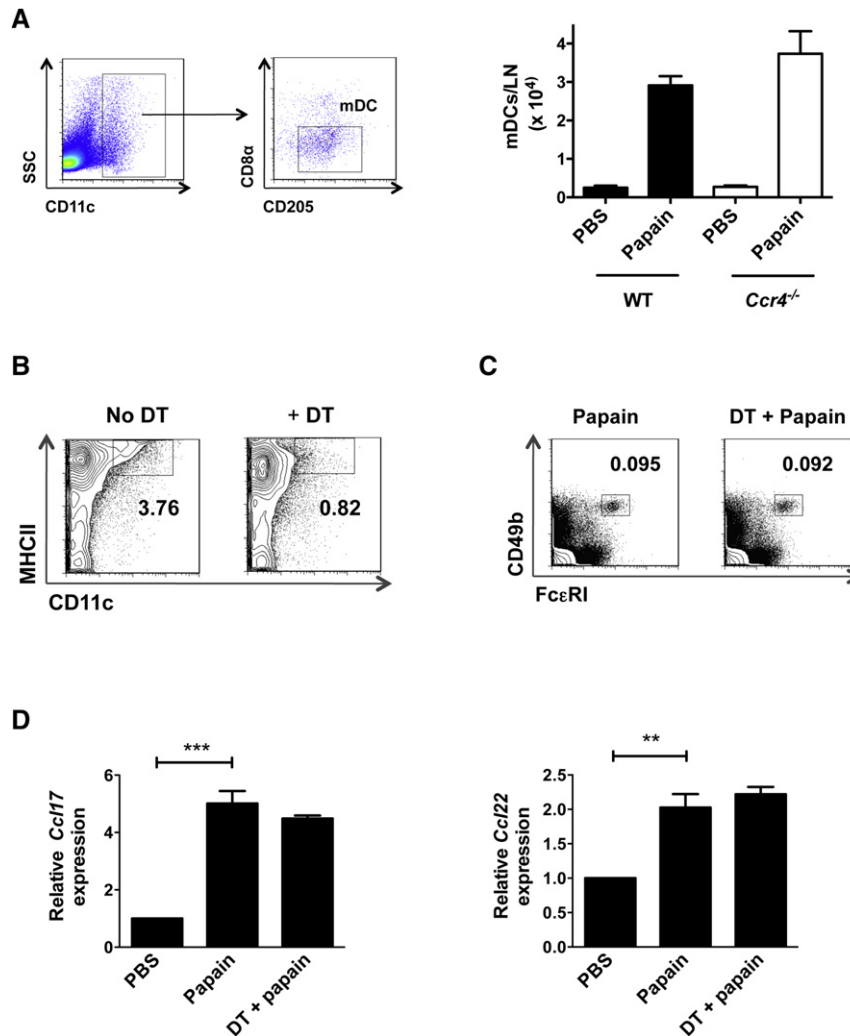


FIG E5. Role of DCs in the pLN immune response to papain immunization. **A**, Migratory dermal DCs (*mDCs*; CD8α⁻CD11c⁺CD205⁺) from pLNs of WT or *Ccr4*^{-/-} mice were enumerated 2 days after PBS or papain immunization. The graph shows means ± SEMs of 2 independent experiments with 2 mice per group. **B**, Total pLN DCs (MHCII^{hi}CD11c⁺) from *Cd11c-Dtr-egfp* transgenic mice 2 days after intraperitoneal injection with PBS or diphtheria toxin (*DT*). **C** and **D**, Basophils (Fig E5, **C**) and *Ccl17* and *Ccl22* gene expression (Fig E5, **D**) in pLNs of mice injected with either PBS or diphtheria toxin before papain immunization. The graph shows means ± SEMs of 2 independent experiments with 2 to 3 mice per group per experiment. ***P* = .003 and ****P* = .0006, 1-way ANOVA.

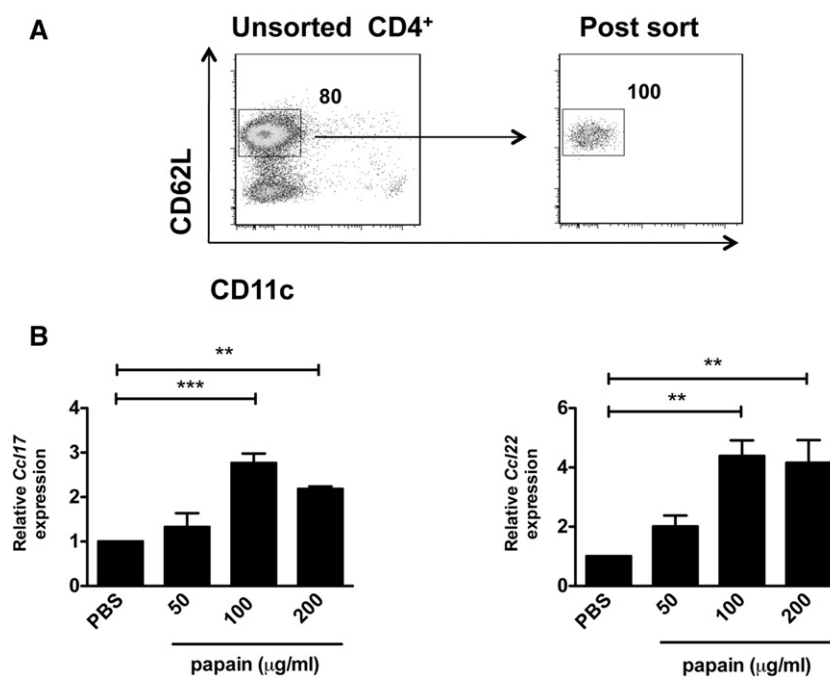


FIG E6. Papain induces chemokine gene expression in purified naive T cells. **A**, Splenic naive T cells ($CD3^+CD4^+CD62L^+CD11c^-$) from unimmunized C57/Bl6 WT mice were isolated by sorting. Numbers above outlined areas indicate percentages of total live cells. **B**, Purified naive T cells were stimulated with PBS or the indicated concentrations of papain *in vitro* for 20 hours, followed by quantification of *Ccl17* and *Ccl22* gene expression. Data are means $\pm$ SEMs of 2 independent experiments with 2 mice per experiment. ** $P = .001$ and *** $P = .0005$, 1-way ANOVA.

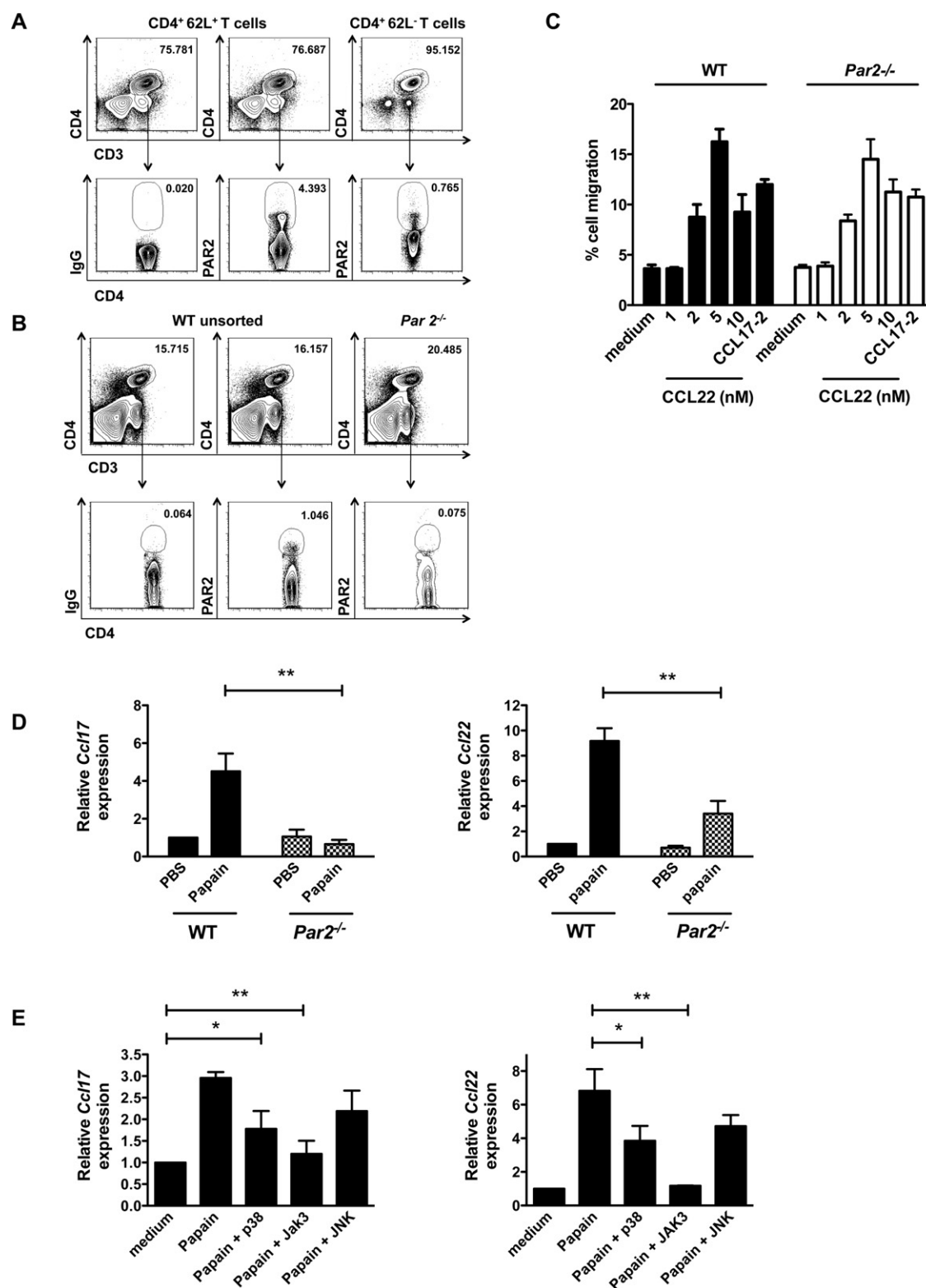


FIG E7. PAR2 expression on T cells and effect of PAR2 deficiency on T-cell responses to papain. **A** and **B**, Naive T cells were isolated from spleens of WT C57Bl/6 or *Par2*^{-/-} mice by means of negative bead selection. Purity of T-cell fractions (top panels) and PAR2 expression (bottom panels) in naive (CD62L⁺; Fig E7, A) and effector/memory (CD62L⁻; Fig E7, B) CD4⁺ T cells. Numbers above outlined areas indicate percentages of total live cells. **C**, Chemotaxis of BMBs from WT or *Par2*^{-/-} mice to the indicated chemokines was evaluated in transwell assays. Data are means ± SEMs of 2 independent experiments with 2 mice per experiment. **D**, Splenic CD4⁺ T cells from WT or *Par2*^{-/-} mice were stimulated with papain (100 μg/mL) for 20 hours *in vitro*, followed by quantification of *Ccl17* and *Ccl22* expression by using real-time PCR. **E**, Splenic

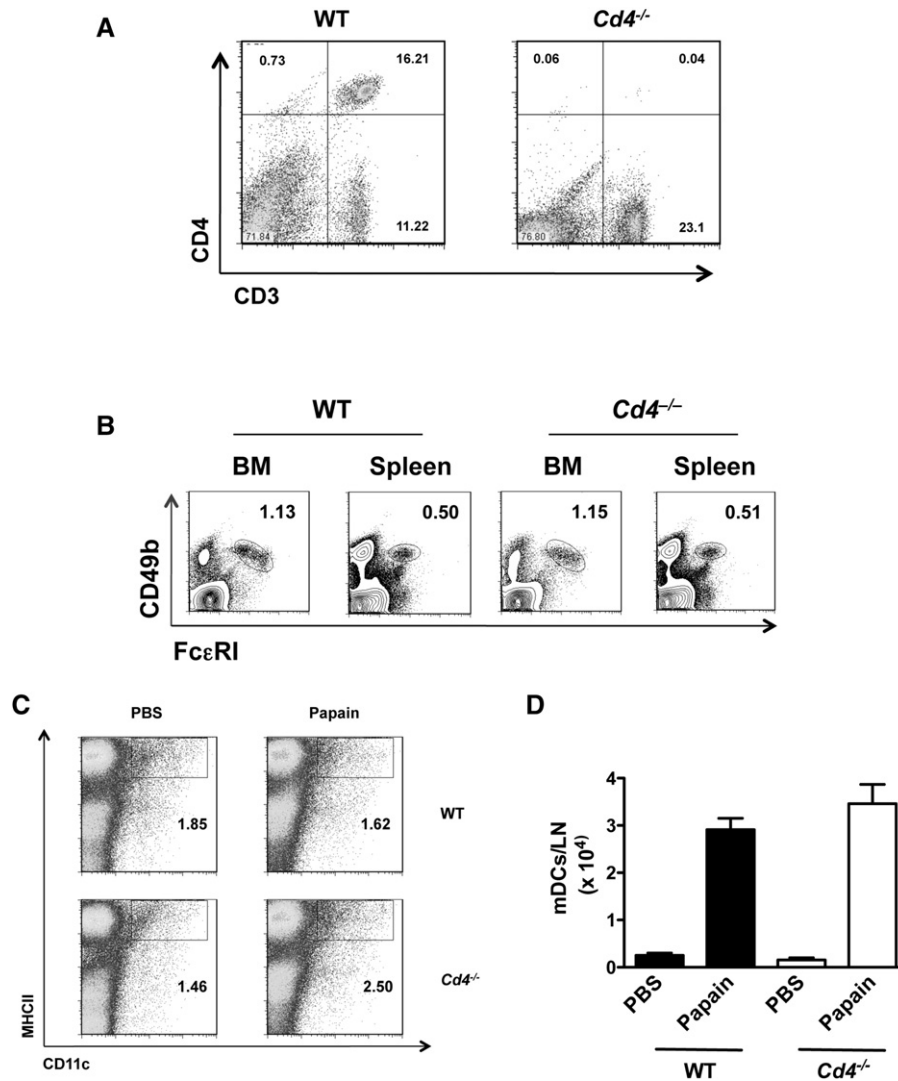


FIG E8. Leukocyte populations in organs of *Cd4*^{-/-} mice. **A**, Absence of CD4⁺ T cells in *Cd4*^{-/-} mice. Total splenocytes from WT C57/Bl6 or *Cd4*^{-/-} mice were evaluated by means of flow cytometry. **B**, Basophil numbers in spleens and freshly isolated BM of naive WT or *Cd4*^{-/-} mice were quantified by using flow cytometry. **C** and **D**, Total DCs (MHCII^{hi}CD11c⁺) or migratory dermal DCs (*mDCs*) in pLNs from WT or *Cd4*^{-/-} mice immunized with PBS or papain. Numbers in each quadrant indicate percentages of total live cells. *Bar graphs* show means ± SEMs of 2 independent experiments with 2 to 3 mice per group per experiment.

CD4⁺ T cells were pretreated with vehicle alone (dimethyl sulfoxide) or the indicated inhibitors for 1 hour, followed by papain stimulation for 20 hours and evaluation of chemokine expression, as in Fig E7, *D*. *Bar graphs* show means ± SEMs of 2 to 3 independent experiments with 2 to 3 mice per group per experiment. **P* = .01 and ***P* = .005, 1-way ANOVA.

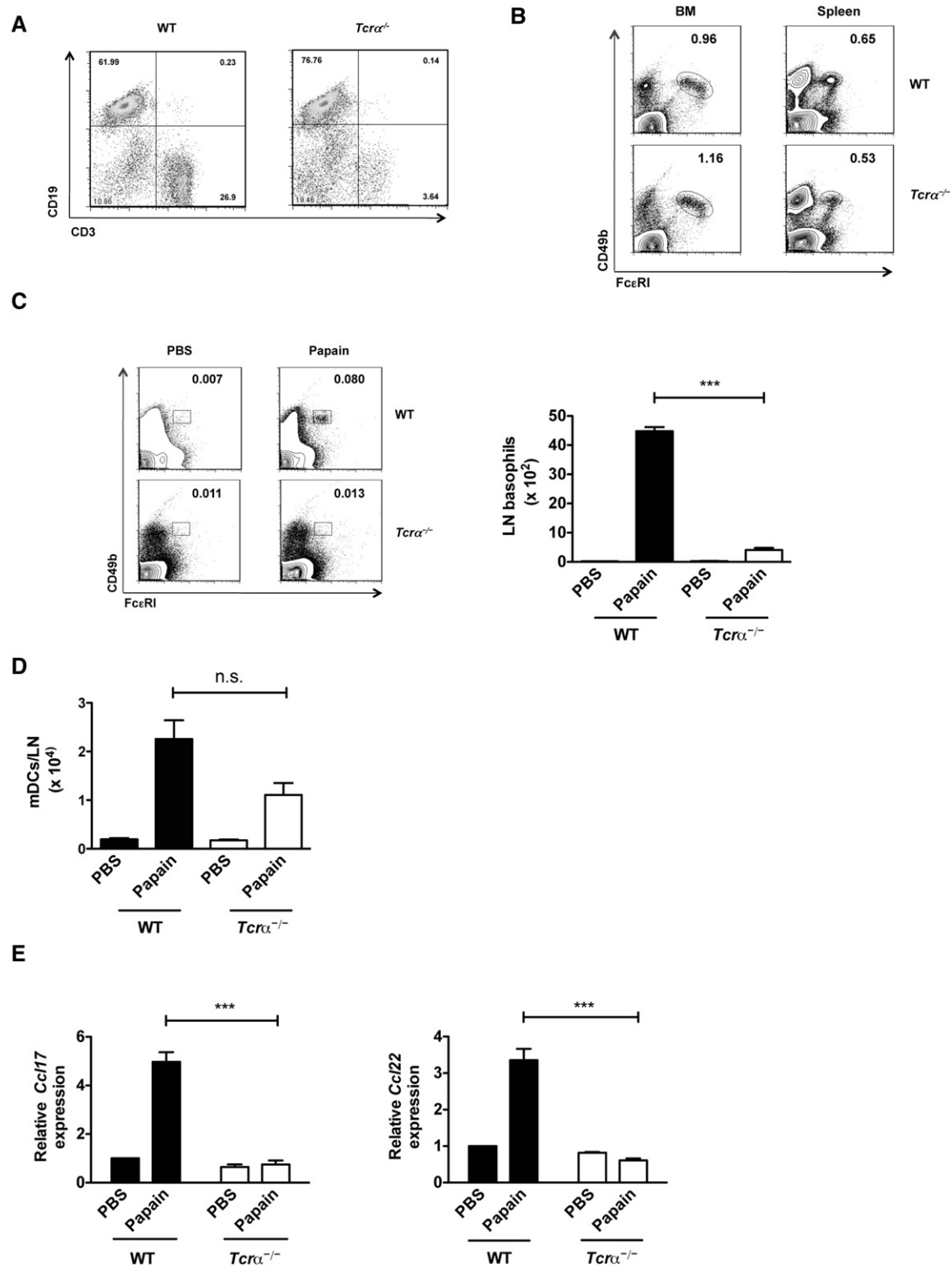


FIG E9. Responses of T-cell deficient (*Tcrα*^{-/-}) mice to papain. **A**, **B** and **T** cells among total splenocytes of WT C57Bl/6 or *Tcrα*^{-/-} mice. Numbers in each quadrant indicate percentages of total live cells. **B**, Basophil numbers in spleens and BM of naive WT or *Tcrα*^{-/-} mice. **C** and **D**, Basophils (Fig E9, **C**) or migratory dermal DCs (*mDCs*; Fig E9, **D**) in pLNs of WT or *Tcrα*^{-/-} mice 3 days after papain immunization. *n.s.*, Not significant. ****P* = .0001, 1-way ANOVA. **E**, *Ccl17* and *Ccl22* expression in pLNs of WT or *Tcrα*^{-/-} mice immunized with PBS or papain determined by using qPCR. ****P* = .0001, 1-way ANOVA. For flow plots, numbers in each quadrant indicate percentages of total live cells. Bar graphs show means ± SEMs of 3 independent experiments with 2 to 3 mice per group per experiment.

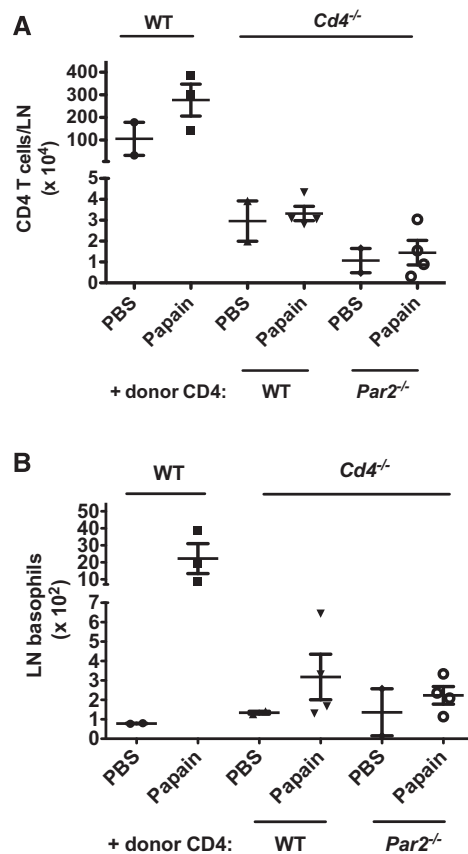


FIG E10. Reconstitution of CD4-deficient mice. *Cd4*^{-/-} mice were injected with CD4⁺ T cells from WT or *Par2*^{-/-} mice intravenously or directly into the rear footpad. Forty-eight hours later, recipient mice were immunized with PBS or papain as in Fig 1, followed by enumeration of CD4⁺ T cells (A) and basophils (B) 2 days later. Data from pLNs of PBS- or papain-immunized WT C57Bl/6 mice that did not receive any donor T cells are presented as a control. Data represent means $\pm$ SEMs of 4 independent experiments with 2 to 3 mice per group per experiment.

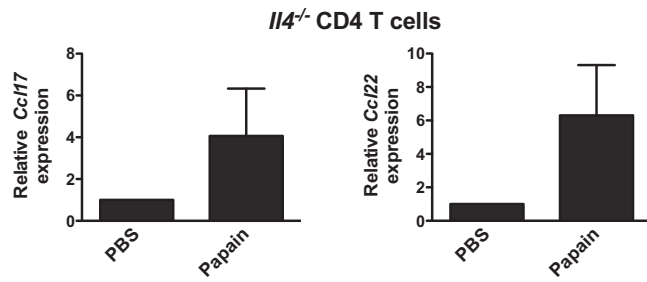


FIG E11. IL-4 is not required for papain-induced chemokine expression in T cells. Splenic CD4⁺ T cells from WT or *Il4*^{-/-} mice were stimulated with papain (100 μ g/mL) for 20 hours *in vitro*, followed by RNA isolation and quantification of *Ccl17* and *Ccl22* gene expression by using real-time PCR. Data are means $\pm$ SEMs of 3 independent experiments with 1 mouse per experiment.

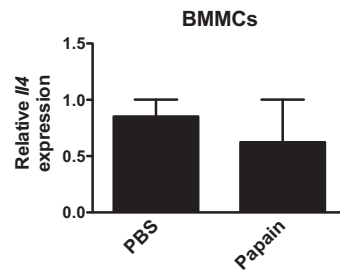


FIG E12. Murine MCs do not upregulate *Il4* expression in response to papain. Bone marrow–derived mast cells (*BMMCs*) were stimulated with papain (100 μ g/mL) for 20 hours *in vitro*, followed by RNA isolation and quantification of *Il4* gene expression by using real-time PCR. Data are means $\pm$ SEMs of 2 independent experiments with cells derived from 2 to 3 mice per experiment.