

Loss of GTPase of immunity-associated protein 5 (Gimap5) promotes pathogenic CD4⁺ T-cell development and allergic airway disease



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Background: GTPase of immunity-associated protein 5 (*GIMAP5*) is essential for lymphocyte homeostasis and survival. Recently, human *GIMAP5* single nucleotide polymorphisms have been linked to an increased risk for asthma, whereas loss of *Gimap5* in mice has been associated with severe CD4⁺ T cell–driven immune pathology.

Objective: We sought to identify the molecular and cellular mechanisms by which *Gimap5* deficiency predisposes to allergic airway disease.

Methods: CD4⁺ T-cell polarization and development of pathogenic CD4⁺ T cells were assessed in *Gimap5*-deficient mice and a human patient with a *GIMAP5* loss-of-function (LOF) mutation. House dust mite–induced airway inflammation was assessed by using a complete *Gimap5* LOF (*Gimap5*^{sph/sph}) and conditional *Gimap5*^{fl/fl}*Cd4*^{Cre/ert2} mice.

Results: *GIMAP5* LOF mutations in both mice and human subjects are associated with spontaneous polarization toward pathogenic T_H17 and T_H2 cells *in vivo*. Mechanistic studies *in vitro* reveal that impairment of *Gimap5*-deficient T_H cell differentiation is associated with increased DNA damage, particularly during T_H1-polarizing conditions. DNA damage in *Gimap5*-deficient CD4⁺ T cells could be controlled by TGF-β, thereby promoting T_H17 polarization. When challenged with house dust mite *in vivo*, *Gimap5*-deficient mice displayed an exacerbated asthma phenotype (inflammation and airway hyperresponsiveness), with increased development of T_H2, T_H17, and pathogenic T_H17/T_H2 cells.

Conclusion: Activation of *Gimap5*-deficient CD4⁺ T cells is associated with increased DNA damage and reduced survival

that can be overcome by TGF-β. This leads to selective survival of pathogenic T_H17 cells but also T_H2 cells in human subjects and mice, ultimately promoting allergic airway disease. (J Allergy Clin Immunol 2019;143:245–57.)

Key words: Allergic airway disease, house dust mite, primary immune deficiency, *GIMAP5*, T_H2/T_H17 cells, TGF-β, DNA damage response, T-cell polarization, immune homeostasis, lung disease

Asthma is a chronic airway inflammatory disorder that develops through a variety of mechanisms, each leading to different clinical manifestations and responsiveness to treatment. Although the majority of asthma cases can be controlled with standard treatments, a subset of asthmatic patients have “severe” or “refractory” asthma.¹ Recent work has shown that these cases are associated with the presence of T_H2/T_H17 CD4⁺ T cells and that these cells drive more severe disease than classical T_H2 CD4⁺ T cells.^{2–4} Moreover, levels of IL-17A or IL-17A–producing CD4⁺ cells in the lung, sputum, and blood of asthmatic patients correlate with disease severity.^{2–11} However, both genetic and environmental factors that contribute to T_H2- and T_H17-associated asthma development remain unclear.

GTPase of immunity-associated protein 5 (*GIMAP5*) is critical for lymphocyte survival, with *Gimap5*-deficient CD4⁺ T cells demonstrating impaired survival and increased DNA damage on antigenic stimulation.^{12–14} Complete loss-of-function (LOF) mutations in *GIMAP5*, both in rodents and in human subjects, result in lymphopenia, loss of immunologic tolerance, and subsequent development of autoimmunity.^{12–21} In the context of *Gimap5*-deficient (*Gimap5*^{sph/sph}) mice, this manifests as CD4⁺ T cell–driven colitis. Importantly, polymorphisms in *GIMAP5* have been associated with increased risk for type 1 diabetes and systemic lupus erythematosus, and a recent study revealed a single nucleotide polymorphism (SNP) in the promoter region of *GIMAP5* to be associated with asthma.^{22–25} However, it remains unclear how polymorphisms in *GIMAP5* predisposes subjects to these inflammatory diseases.

In the current study we show that *Gimap5* deficiency drives the development of pathogenic CD4⁺ T cells with an exaggerated T_H17 cell response and a more severe asthma phenotype.

METHODS

Study design

For the proposed experiments involving studies of immune responses *in vivo*, both male and female mice were used. All mice used were generated on a C57BL/6J background, and background purity was confirmed by using whole-genome SNP analysis. CD4⁺ T cells from 3- to 4-week-old mice were used, unless otherwise noted, to minimize confounding by

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Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
cKO:	Conditional knockout
Foxp3:	Forkhead box P3
GIMAP5:	GTPase of Immunity-associated protein 5
GSK3:	Glycogen synthase kinase 3
γ H2AX:	Phosphorylated histone H2AX
HDM:	House dust mite
ILC:	Innate lymphoid cell
iTreg:	Induced regulatory T
LOF:	Loss of function
mLN:	Mesenteric lymph node
NFATc1:	Nuclear factor of activated T cells 1
PMA:	Phorbol 12-myristate 13-acetate
ROR γ t:	Retinoic acid–related orphan receptor γ t
SNP:	Single nucleotide polymorphism
TF:	Transcription factor
Treg:	Regulatory T
WT:	Wild-type

secondary factors arising from lymphopenia and other late-stage pathologies that develop in *Gimap5^{sph/sph}* mice. At this age, *Gimap5^{sph/sph}* mice have relatively normal numbers of CD4⁺ T cells, with a normal frequency of naive T cells that exhibit a quiescent phenotype comparable with that of wild-type (WT) mice. Because we were interested in T-cell proliferation/survival in WT versus *Gimap5*-deficient cells, we expected to find strong differences in measurement (eg, normal vs absence of T-cell survival/proliferation). In cases in which the differences in the observed phenomena were clear and distinct, a group size of 6 mice was suitable to produce statistically significant (ie, $P < .05$) data. For these studies, we estimated that, with a sample size of 6, we have a 99% power to detect at least 25% reduction in the percentage of proliferating cells in stimulated *Gimap5^{sph/sph}* cells with a significant level (α) of .05 (2-tailed).

Mice and reagents

All experiments were performed according to US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital. C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Me). Deletion of *Gimap5* in *Gimap5^{flox/flox}Cd4^{cre-ert2}* mice was induced by administration of tamoxifen chow (40 mg/kg body weight; Teklad Diets; Harlan Laboratories, Indianapolis, Ind). *Gimap5^{sph/sph}* mice were generated, as previously described,⁶ and bred in house in the vivarium of Cincinnati Children's Hospital. All mice were maintained under specific pathogen-free conditions.

All antibodies used for flow cytometry were purchased from eBioscience (San Diego, Calif) or BioLegend (San Diego, Calif), unless otherwise noted. Purified α -mouse CD3 (17A2) and α -mouse CD28 (37.51) antibodies (BioLegend) were used for mouse T-cell activation. A fixable viability stain was purchased from eBioscience. 7-Aminoactinomycin D was purchased from BD (Franklin Lakes, NJ). Phorbol 12-myristate 13-acetate (PMA), ionomycin, and Brefeldin A were obtained from Sigma (St Louis, Mo). α IFN- γ (XMG1.2) and mIL-23 were obtained from eBioscience. TGF- β 1, mIL-4, and hIL-2 were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). α IL-4, IL-6, and mIL-1 β , mIL-12, and TGF- β 3 were obtained from PeproTech (Rocky Hills, NJ).

Generation of *Gimap5^{flox/flox}* mice

CRISPR/Cas9 was used to insert LoxP sites flanking the second exon of *Gimap5* (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Because the second exon contains the translation start site, deletion of

this exon is expected to result in loss of *Gimap5* protein (see Fig E2, A, in this article's Online Repository at www.jacionline.org). The accuracy of the insert was confirmed through sequencing (see Fig E1, B). The presence of the LoxP sites was confirmed through PCR genotyping, as shown in Fig E1, C. The PCR primers used were as follows: 5' LoxP site PCR forward, 5'-GATC GTGCAACGACATGGG-3'; 5' LoxP site PCR reverse, 5'-ACG ACCTTGCCCCGACTAGAG-3'; 3' LoxP site PCR forward, 5'-CCACC CAAGGAGAGTTACCC-3'; and 3' LoxP site PCR reverse, 5'-GTCAAAGA CAAGCTTCCACAGT-3'. Mice bearing the *Gimap5^{flox/flox}* allele were then crossed with those carrying the *Cd4^{cre-ert2}* and *Foxp3^{cre}* transgenes to produce *Gimap5^{flox/flox}Cd4^{cre-ert2}* or *Gimap5^{flox/flox}Foxp3^{cre}* offspring. Deletion of *Gimap5* was induced in *Gimap5^{flox/flox}Cd4^{cre-ert2}* CD4⁺ T cells through administration of tamoxifen chow (40 mg/kg body weight; Teklad Diets; Harlan Laboratories).

Flow cytometry and T-cell analyses

T-cell proliferation was quantified by incubating Mojo-purified (BioLegend) CD4⁺ T cells in 5 μ mol/L CellTrace Violet (CTV) in 0.2% FBS for 20 minutes. Cells were cultured in supplemented Iscove modified Dulbecco medium containing 10% FBS, 2% penicillin/streptomycin, 1% L-glutamine, and 50 μ mol/L β -Mercaptoethanol (BME). Cells were either left unstimulated or were stimulated with α CD3 (1 μ g/mL)/ α CD28 (2 μ g/mL) in the presence of TGF- β 1 (2 ng/mL). After 3 days of incubation, proliferation was evaluated by analyzing CTV dilution by using flow cytometry. Viability was determined by staining with a fixable viability dye. DNA damage was evaluated by means of phosphorylated histone H2AX (γ H2AX) staining in conjunction with intracellular 7-aminoactinomycin D staining for cell-cycle analysis. Lymphocytes from the spleens, mesenteric lymph nodes (mLNs), mediastinal lymph nodes, and lungs were isolated and stained with fluorochrome-conjugated antibodies for mouse CD3, CD4, CD19, CD44, γ H2AX, T-bet, retinoic acid–related orphan receptor γ t (ROR γ t), Gata-3, and forkhead box P3 (Foxp3) to evaluate lymphocyte populations *ex vivo*. Lymphocytes from the spleens, mLNs, mediastinal lymph nodes, and lungs were restimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of Brefeldin A for 6 hours before staining with fluorochrome-conjugated antibodies for mouse IFN- γ , IL-13, and IL-17A to evaluate CD4⁺ T-cell cytokine production capacity. Except where noted in *in vitro* experiments, all data were generated from live cells, as determined by using fixable viability dye staining.

In vitro CD4⁺ T-cell polarization

Mojo-purified (BioLegend) naive CD4⁺ T cells were isolated from spleens and peripheral lymph nodes (cervical, brachial, axillary, and inguinal) of 3- to 4-week-old mice and labeled with 5 μ mol/L CTV in 0.2% FBS for 20 minutes to test CD4⁺ T-cell polarization. Cells were cultured in supplemented RPMI media containing 10% FBS, 2% penicillin/streptomycin, and 50 μ mol/L BME. Cells were stimulated with α CD3 (1 μ g/mL)/ α CD28 (2 μ g/mL) alone or in the presence of polarizing cytokines (see Table E1 in this article's Online Repository at www.jacionline.org).

Mouse asthma model

Because *Gimap5^{sph/sph}* mice show progressive changes in differentiated CD4⁺ T cells with age,^{12,14} intratracheal injections started at 4 weeks of age, when a similar frequency of naive CD4⁺ T cells can be observed compared with that in WT littermates.^{12,14} WT C57BL/6 and *Gimap5^{sph/sph}* mice were administered nine 25- μ L intratracheal injections of 25 μ g of house dust mite (HDM) or saline over 3 weeks. For older (6–8 weeks) WT, *Gimap5^{flox/flox}Cd4^{cre-ert2}*, and *Gimap5^{flox/flox}Foxp3^{cre}* mice, doses of 50 μ g of HDM in 50 μ L of saline were used. WT and *Gimap5^{flox/flox}Cd4^{cre-ert2}* mice were started on tamoxifen chow (40 mg/kg body weight; Teklad Diets; Harlan Laboratories) 7 days before the first intratracheal injection in relevant experiments. In HDM experiments mice were killed 24 hours after the last exposure.

Airway hyperresponsiveness

Mice were anesthetized with ketamine, xylazine, and acepromazine (100, 20, and 10 mg/mL, respectively, mixed at a ratio of 4:1:1). Invasive measurements of airway responsiveness were made with the flexiVent apparatus (SCIREQ, Montreal, Quebec, Canada). Mouse tracheas were cannulated with a 19-gauge blunt needle; mice were ventilated at 150 breaths/min and 3.0 cm of water positive end-expiratory pressure. Two total lung capacity perturbations were then performed for airway recruitment before baseline measurement, and subsequent methacholine challenges were performed. Dynamic resistance was assessed after exposure to increasing concentrations of aerosolized methacholine (0, 12.5, 25, 50, and 100 mg/mL). The average of the 3 highest dynamic resistance values with a coefficient of determination of 0.9 or greater (as determined by flexiVent software) was used to determine the dose-response curve.

Bronchoalveolar lavage fluid cell collection and analysis

Bronchoalveolar lavage (BAL) was performed by means of tracheal cannulation. Lungs were lavaged with 1 mL of PBS. After counting the number of cells with a hemocytometer, cells were spun onto slides and stained with the HEMA3 stain set (Fisher Scientific, Waltham, Mass). After deidentifying samples, approximately 200 cells were counted, and numbers of macrophages, neutrophils, and eosinophils were calculated.

Isolation and characterization of lung-infiltrating cells

After lavage, lungs were removed, and the upper right lobe was minced and digested in 2 mL of RPMI plus 0.5 mg/mL Liberase DL (Roche Diagnostics, Mannheim, Germany) and 0.5 mg/mL DNase I (Sigma) at 37°C for 30 minutes. Cells were then passed over a 70- μ m cell strainer. Cells were washed with PBS and counted. Approximately 1×10^6 cells were stimulated with 50 ng/mL PMA plus 500 ng/mL ionomycin for 6 hours. After 2 hours, Brefeldin A was added. CD4⁺ T cells were stained with a viability dye (eBioscience Live/Dead stain) and antibodies for CD3, CD4, CD44, and $\gamma\delta$ T-cell receptor; intracellular staining for IL-13, IL-17A, and IFN- γ was performed after fixation and permeabilization, according to the manufacturer's instructions (eBioscience). Foxp3 staining was performed on separate cells that had not been restimulated *ex vivo*.

Histology

Lung tissue was collected and immediately fixed in 10% buffered formalin solution, followed by routine paraffin embedding. Periodic acid-Schiff and hematoxylin and eosin staining was performed on 4- μ m sections from the paraffin-embedded tissue blocks for conventional light microscopy.

Analysis of human T cells

For all studies concerning human cells, informed consent was obtained, and studies were approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. PBMCs were isolated from whole blood of a GIMAP5-deficient patient, a healthy parent (heterozygous for GIMAP5 rs72650695), and unrelated healthy control subjects by using Ficoll-Paque Plus density gradient centrifugation. Isolated PBMCs were cultured in RPMI, 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 10 mmol/L HEPES and were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of Brefeldin A and analyzed for IFN- γ , IL-17A, and IL-13 production by using flow cytometry.

Immunoblotting

Protein lysates from Mojo-isolated (BioLegend) CD4⁺ T cells were prepared according to standard methods from resting cells. Lysates were separated by using 10% Bis-Tris Gels, transferred onto nitrocellulose, and

immunoblotted with primary antibodies to Gimap5 (MAC421)¹² and β -actin (CST).

Analysis and graphics software

Flow cytometric data were analyzed by using FlowJo software (TreeStar, Ashland, Ore). All data were analyzed for statistical significance by using GraphPad Prism 4 software (GraphPad Software, San Diego, Calif). The graphical abstract was created with BioRender. Figure components were generated in FlowJo or GraphPad Prism software (GraphPad Software, La Jolla, Calif) and assembled in Adobe Illustrator (Adobe, San Jose, Calif).

Statistical analysis

Data are expressed as means $\pm$ SDs, except for where otherwise noted. For studies comparing T cells from C57BL/6J, *Gimap5^{sph/sph}*, and/or *Gimap5^{fl/fl} Cd4^{cre-ert2}* mice, the Student 2-tailed test or ANOVA followed by the Sidak multiple comparisons test for 3 or more groups were used. Data were considered statistically significant if *P* values were less than .5.

RESULTS

GIMAP5 LOF mutations in mice and human subjects promote development of T_H17/T_H2 CD4⁺ T cells *in vivo*

Given that polymorphisms in GIMAP5 have been associated with increased risk for type 1 diabetes, systemic lupus erythematosus, and asthma²²⁻²⁵ and our previous studies revealed the development of CD4-dependent colitis in Gimap5-deficient mice,^{14,15} we first investigated whether baseline differences in CD4⁺ T-cell polarization exist *in vivo*. To assess this, we examined the CD4⁺ T-cell compartment in the spleens and mLNs of 8-week-old WT and *Gimap5^{sph/sph}* mice. As previously described, CD4⁺ T cells were reduced in the spleens of *Gimap5^{sph/sph}* mice (Fig 1, A). Importantly, however, an increased proportion of the remaining *Gimap5^{sph/sph}* CD4⁺ T cells were polarized to T_H2 cells (Gata-3⁺CD4⁺) and T_H17 cells (ROR γ t⁺CD4⁺) but not T_H1 (T-bet⁺CD4⁺) or regulatory T (Treg) cells (Foxp3⁺CD4⁺) compared with WT mice (Fig 1, B). Furthermore, *Gimap5^{sph/sph}* splenic CD4⁺ T cells more frequently produced IFN- γ , IL-17A, and/or IL-13 compared with WT CD4⁺ T cells (Fig 1, C-E). Notably, *Gimap5^{sph/sph}* mice had expanded frequencies of CD4⁺ T cells that produced both IFN- γ ⁺IL-17A⁺ or IL-13⁺IL-17A⁺ (Fig 1, F and G). These cells have been associated with pathology in patients with several autoimmune diseases and asthma, respectively.^{2,3,26-31}

Interestingly, the mLNs of *Gimap5^{sph/sph}* mice contained similar numbers of total CD4⁺ T cells compared with WT littermates, but a greater frequency expressed T-bet, Gata-3, or ROR γ t compared with WT littermates (Fig 1, H and I). Similar to the spleen, an increased proportion of CD4⁺ T cells in the mLNs were IFN- γ ⁺, IL-17A⁺, IL-13⁺, and the rarer IFN- γ ⁺IL-17A⁺ double producers (Fig 1, J-N). Together, these observations suggest that loss of Gimap5 is associated with an increased polarization of effector CD4⁺ T cells, including development of pathogenic CD4⁺ T cells that can drive immunopathology.

Previously, we described a patient with an LOF mutation in GIMAP5 (rs72650695; a missense variant leading to a Leu204 $\rightarrow$ Pro amino acid change and a complete loss of protein expression),¹⁴ resulting in an immunodeficiency with features of autoimmunity. Functional characterization of CD4⁺ T cells from this patient revealed strikingly similar molecular defects to

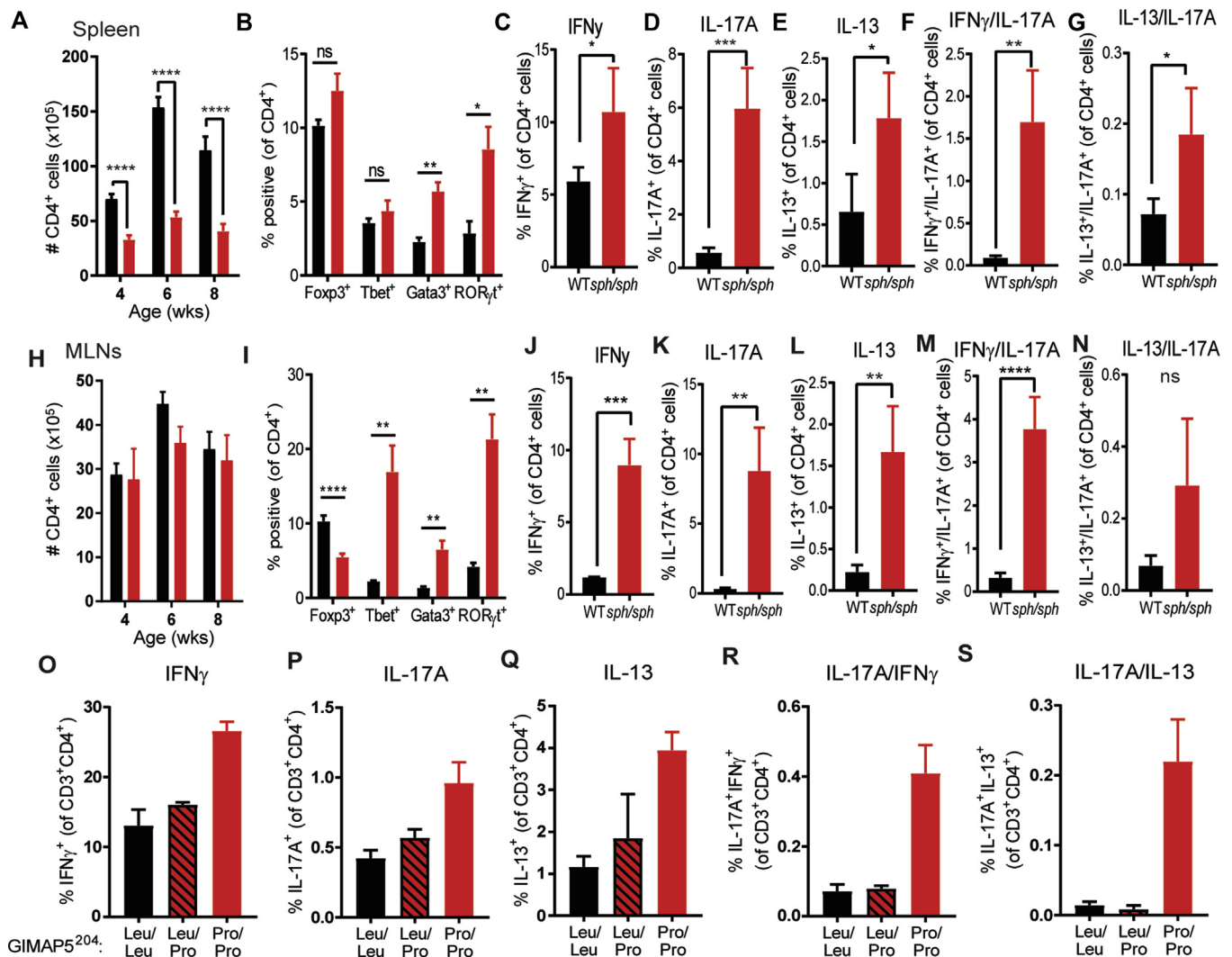


FIG 1. Enhanced *In vivo* polarization of *Gimap5*-deficient mouse and human CD4⁺ T cells. **A**, Number of CD4⁺ T cells in the spleens of 8-week-old WT (black bars) and *Gimap5^{sp/ssh}* (red bars) mice. **B**, Frequency of splenic CD4⁺ T cells expressing Foxp3, Tbet, Gata-3, or RORγt (n = 7). **C–G**, Expression of IFN-γ, IL-17A, and/or IL-13 in splenic CD4⁺ T cells after PMA/ionomycin stimulation (n = 4). **H**, Number of CD4⁺ T cells in the MLNs of 8-week-old mice. **I**, Frequency of CD4⁺ T cells expressing Foxp3, Tbet, Gata-3, or RORγt in the MLNs (n = 7). **J–N**, Expression of IFN-γ, IL-17A, and/or IL-13 in CD4⁺ T cells from the MLNs after PMA/ionomycin stimulation (n = 4). Bars represent mean values ± SDs. Statistical significance is determined by using the Student 2-tailed test. **O–S**, Frequency of CD4⁺ T cells producing IFN-γ, IL-17A, and/or IL-13 on PMA/ionomycin stimulation. T cells isolated from the PBMCs of a *GIMAP5^{204-Pro/Pro}* patient (*GIMAP5^{204-Pro/Pro}*), his heterozygous parent, and unrelated control subjects (n = 2, 2, and 7, respectively) are shown. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. ns, Not significant.

Gimap5-deficient mouse CD4⁺ T cells.¹⁴ Therefore we asked whether the patient has similarly skewed the CD4⁺ T-cell compartment. Strikingly, a greater proportion of the patient's circulating CD4⁺ T cells expressed IL-17A, IL-13, or IFN-γ compared with the patient's heterozygous mother or unrelated control subjects on stimulation with PMA and ionomycin (Fig 1, O–Q). Furthermore, an increased frequency of CD4⁺ T cells coexpressed IL-17A⁺IL-13⁺ or IL-17A⁺IFN-γ⁺ (Fig 1, R and S). Although we were only able to study a single patient with *GIMAP5* deficiency, the increased development of pathogenic peripheral T_H2 and T_H17 cells in the *GIMAP5*-deficient patient is consistent with our mouse data.

Notably, this predilection toward T_H17 polarization can be observed in *in vitro* culture systems. In previous studies we observed that bulk *Gimap5^{sp/ssh}* CD4⁺ T cells have an impaired proliferation and survival on stimulation with αCD3/αCD28 (Fig 2, A and B), and only a small subset is able to survive and proliferate.^{12,14} A more in-depth evaluation of surviving cells demonstrates expression of high levels of RORγt, indicating either a selective survival of T_H17-polarized cells or a predisposition of surviving cells to adopt a T_H17 (Fig 2, C). Together, these data suggest that *GIMAP5* deficiency in both human subjects and mice causes CD4⁺ T cells to preferentially adopt a T_H17 phenotype both *in vitro* and *in vivo*.

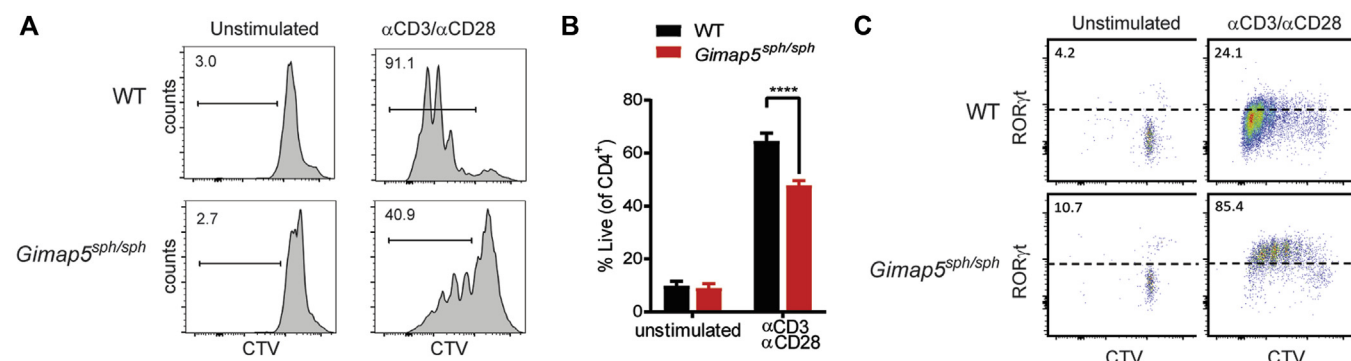


FIG 2. Preferential expression of ROR γ t by surviving *Gimap5*^{sp^h/sp^h CD4⁺ T cells on T-cell receptor stimulation. **A**, Proliferation of WT and *Gimap5*^{sp^h/sp^h CD4⁺ T cells after 3 days of stimulation with α CD3 (1 μ g/mL) plus α CD28 (2 μ g/mL). **B**, Survival of CD4⁺ T cells after 3 days of stimulation. **C**, Expression of ROR γ t in live CD4⁺ T cells after 3 days of stimulation with α CD3/ α CD28. Plots depict live cells (Fig 2, C), whereas bar graphs (Fig 2, B) represent mean values $\pm$ SDs ($n = 4$). All experiments were performed at least 3 times. Statistical significance is determined by using the Student 2-tailed test. **** $P < .0001$.}}

Selective survival of *Gimap5*-deficient CD4⁺ T cells under different polarizing conditions

Both *Gimap5*-deficient rodents and human subjects have lymphopenia,^{12–19} and thus it is unclear whether the increased prevalence of T_H17 cells *in vivo* is due to a skewing of polarization or selective survival of T_H17 cells. Similarly, expression of ROR γ t by almost all surviving *Gimap5*-deficient CD4⁺ T cells could be due to the survival of cells or merely the consequence of surviving cells polarizing to T_H17 cells *in vitro*.

Naive CD4⁺ T cells were isolated from 3- to 4-week-old WT and *Gimap5*^{sp^h/sp^h mice and stimulated with α CD3/ α CD28 in T_H0-, T_H1-, T_H2-, T_H17-, or induced regulatory T (iTreg)-polarizing conditions for 3 days (as detailed in Table E1) before evaluating survival and proliferation to differentiate between these possibilities. As with total CD4⁺ T cells, naive *Gimap5*^{sp^h/sp^h CD4⁺ T cells exhibited reduced survival and proliferation when stimulated with α CD3/ α CD28 (T_H0 conditions) compared with WT CD4⁺ T cells (Fig 3, A and B). A similar defect was observed in T_H1-polarizing conditions. This defect was primarily characterized by *Gimap5*^{sp^h/sp^h cells unable to complete the cell cycle, although a considerable proportion of cells died after cycling. Stimulation in T_H2-polarizing conditions resulted in a more bifurcated response, with a subset of cells exhibiting strong proliferation, whereas a significant number of cells died without completing the cell cycle. Strikingly, compared with WT mice, naive *Gimap5*^{sp^h/sp^h CD4⁺ T-cell survival and proliferation were completely normal in T_H17- and iTreg-polarizing conditions.}}}}

Notably, both T_H17- and iTreg-polarizing conditions include TGF- β 1; therefore we asked whether TGF- β signaling promoted *Gimap5*^{sp^h/sp^h CD4⁺ T-cell survival and proliferation during T_H17 polarization. Previously, Lee et al³² identified different T_H17-polarizing conditions by using combinations of TGF- β 1, TGF- β 3, IL-6, IL-23, and IL-1 β (see Table E1).}

To test the contribution of TGF- β and the T_H17 transcriptional program to *Gimap5*^{sp^h/sp^h CD4⁺ T-cell survival, we stimulated naive CD4⁺ T cells in T_H17-polarizing conditions containing TGF- β 1, TGF- β 3, or anti-TGF- β 1. Although *Gimap5*^{sp^h/sp^h CD4⁺ T-cell survival and proliferation were improved in T_H17-polarizing conditions lacking TGF- β signaling over T_H0 conditions, survival was significantly reduced compared with}}

T_H17-polarizing conditions containing TGF- β 1 or TGF- β 3 (Fig 3, C). These data suggest that TGF- β is a critical mediator of *Gimap5*-deficient CD4⁺ T-cell survival, ultimately promoting the development of pathogenic T_H17 cells.

Activation of *Gimap5*^{sp^h/sp^h CD4⁺ T cells results in increased DNA damage that is mitigated by TGF- β 1}

We have previously reported that poor survival of *Gimap5*^{sp^h/sp^h CD4⁺ T cells on α CD3/ α CD28 stimulation correlated with exacerbated DNA damage.¹⁴ Therefore we asked whether the reduced survival in different polarizing conditions was associated with exacerbated DNA damage. Specifically, we assessed the level of γ H2AX in live dividing CD4⁺ T cells as a marker for double-stranded DNA breaks. Interestingly, high levels of γ H2AX were observed, even in WT CD4⁺ T cells, when stimulated under T_H0, T_H1, or T_H2 conditions, whereas T_H17 and iTreg conditions were associated with comparatively modest DNA damage after 3 days of polarizing conditions (Fig 3, D). Importantly, *Gimap5*^{sp^h/sp^h CD4⁺ T cells consistently showed increased DNA damage in T_H0, T_H1, or T_H2 conditions compared with WT conditions (Fig 3, D), although γ H2AX levels in both WT and *Gimap5*^{sp^h/sp^h CD4⁺ T cells were reduced in polarizing conditions containing either TGF- β 1 or TGF- β 3. Notably, increased DNA damage was also observed in *Gimap5*^{sp^h/sp^h T_H1 (T-bet⁺) cells directly *ex vivo*, indicating that this phenotype remains relevant *in vivo* (see Fig E3 in this article's Online Repository at www.jacionline.org). Together, these data indicate that *Gimap5*^{sp^h/sp^h CD4⁺ T cells are unable to control the DNA damage that occurs in T_H0-, T_H1-, and T_H2-polarizing conditions.}}}}}

Given the preferential survival of *Gimap5*-deficient CD4⁺ T cells stimulated in TGF- β -containing T_H17- and iTreg-polarizing conditions, we next asked whether TGF- β 1 signaling was sufficient to rescue CD4⁺ T cells cultured in polarizing conditions associated with poor survival. Strikingly, proliferation and survival of *Gimap5*^{sp^h/sp^h CD4⁺ T cells were almost completely rescued with the addition of TGF- β 1 in cells with these polarizing conditions (Fig 4, A and B).}

Notably, TGF- β is cleaved to its active form in the lung (and other tissues) after tissue injury,^{33–36} resulting in a TGF- β -rich environment. Because naive T cells are first activated in lymph

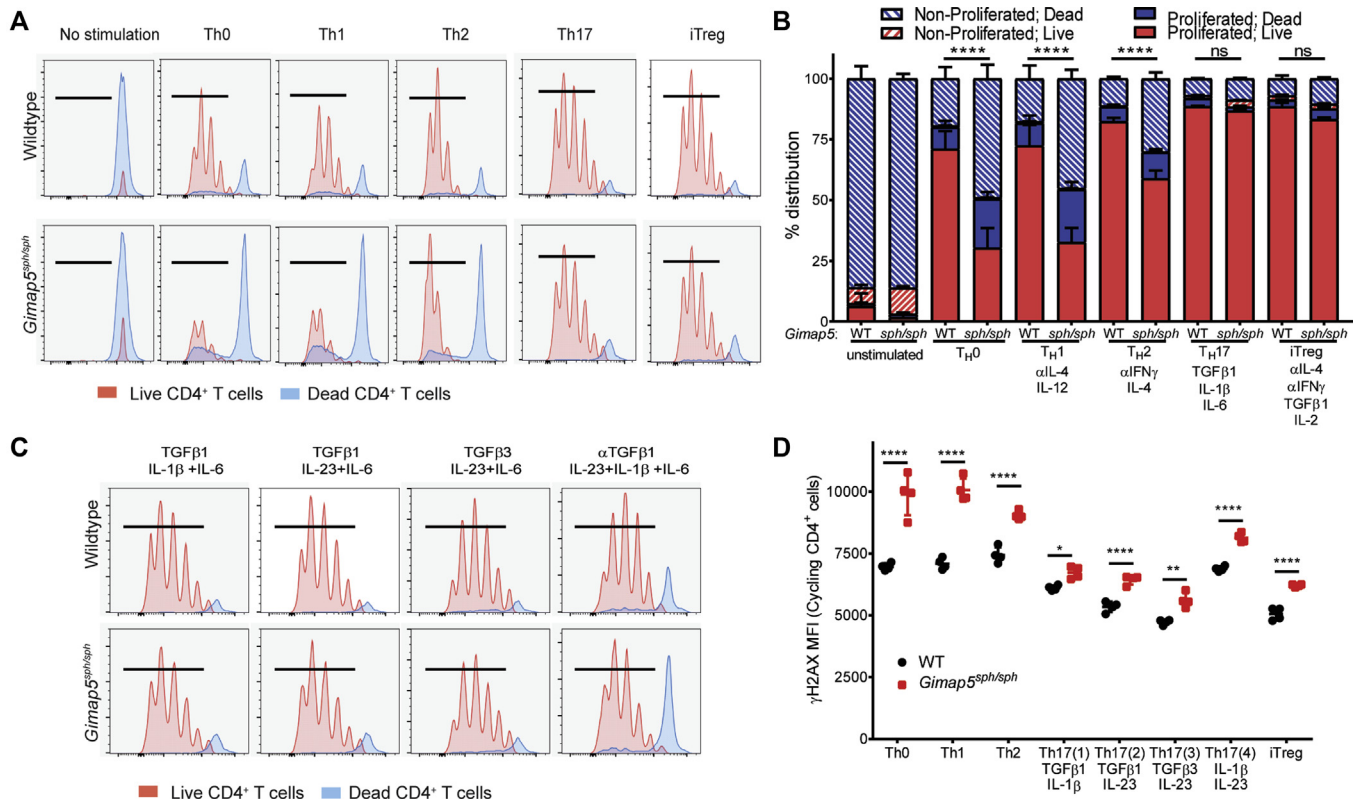


FIG 3. Preferential survival of *Gimap5^{sp/sp}* CD4⁺ T cells in Th17- and iTreg-polarizing conditions is associated with TGF-β. **A** and **B**, Proliferation and survival of naive CD4⁺ T cells from WT and *Gimap5^{sp/sp}* mice stimulated for 3 days in nonpolarizing conditions (Th0) or Th1-, Th2-, Th17-, or iTreg-polarizing conditions. Blue histograms represent dead cells; red histograms show live cells. Hatched bars represent nonproliferated cells; solid bars represent proliferated cells. **C**, Proliferation of live/dead CD4⁺ T cells after 3 days of stimulation in different Th17-polarizing conditions. **D**, γH2AX expression (DNA damage) in live CD4⁺ T cells in cycle after 3 days of stimulation in different polarizing conditions. Data represent mean values ± SDs (n = 4). All experiments were performed at least 3 times. Statistical significance is determined by means of ANOVA, followed by using the Sidak multiple comparisons test. *P < .05, **P < .01, ***P < .001, and ****P < .0001. ns, Not significant.

nodes before migrating to the affected tissue, we asked whether exposure to TGF-β1 during the cycling phase, when DNA damage is most pronounced, was sufficient to promote survival of *Gimap5^{sp/sp}* CD4⁺ T cells. Indeed, the addition of TGF-β1 after 1 or 2 days of stimulation/polarization robustly improved *Gimap5^{sp/sp}* CD4⁺ T-cell survival, proliferation, and control of DNA damage in Th0-, Th1-, and Th2-polarizing conditions, although it was insufficient to completely abrogate the effects of *Gimap5* deficiency (Fig 4, C-H). These data demonstrate that, independent of Th17 or iTreg polarization, TGF-β1 is sufficient to rescue the survival of *Gimap5^{sp/sp}* CD4⁺ T cells. Moreover, TGF-β1 signaling is not required at the time of initial αCD3/αCD28 stimulation. Thus these studies identify TGF-β as an important mediator during CD4⁺ T-cell differentiation, limiting DNA damage and improving survival and proliferation of activated *Gimap5^{sp/sp}* CD4⁺ T cells and promoting the selective development of pathogenic Th17 cells.

Gimap5 deficiency exacerbates allergic airway pathology

A recent study identifies *GIMAP5* as a modifier gene in asthmatic patients.²⁵ Specifically, a 5' SNP (rs6965571) in the

promoter region of *GIMAP5* is associated with an approximately 3-fold increased risk of asthma in a prospective Swedish birth cohort,²⁵ although the mechanisms responsible for increased asthma risk are unclear. Given our findings that both *GIMAP5*-deficient mice and human subjects display a high frequency of Th2 and Th2/Th17 pathogenic CD4⁺ T cells *in vivo* and that Th2/Th17 allergic responses exacerbate the asthma phenotype, we assessed the role of *Gimap5* in allergic airway disease. Based on the changes in T-cell polarization described above, we predicted that loss of *Gimap5* would increase sensitivity to allergic airway disease, and therefore we used a mild model of experimentally induced allergic airway disease in C57BL/6J mice.^{37,38} Specifically, we sensitized *Gimap5^{sp/sp}* and control C57BL/6 WT mice with 9 intratracheal injections of 25 μg of HDM during a 3-week period, as previously described,³⁹ after which immune pathology was assessed. One day after the last exposure, mice were evaluated for airway hyperresponsiveness (AHR) and BAL inflammatory profile. Strikingly, HDM-sensitized *Gimap5^{sp/sp}* mice exhibited significantly greater AHR compared with HDM-sensitized WT mice and saline-treated mice (Fig 5, A). The observed increase in AHR corresponded with a significantly increased infiltration of eosinophils and macrophages in the BAL fluid in HDM-sensitized *Gimap5^{sp/sp}* mice (Fig 5, B-D).

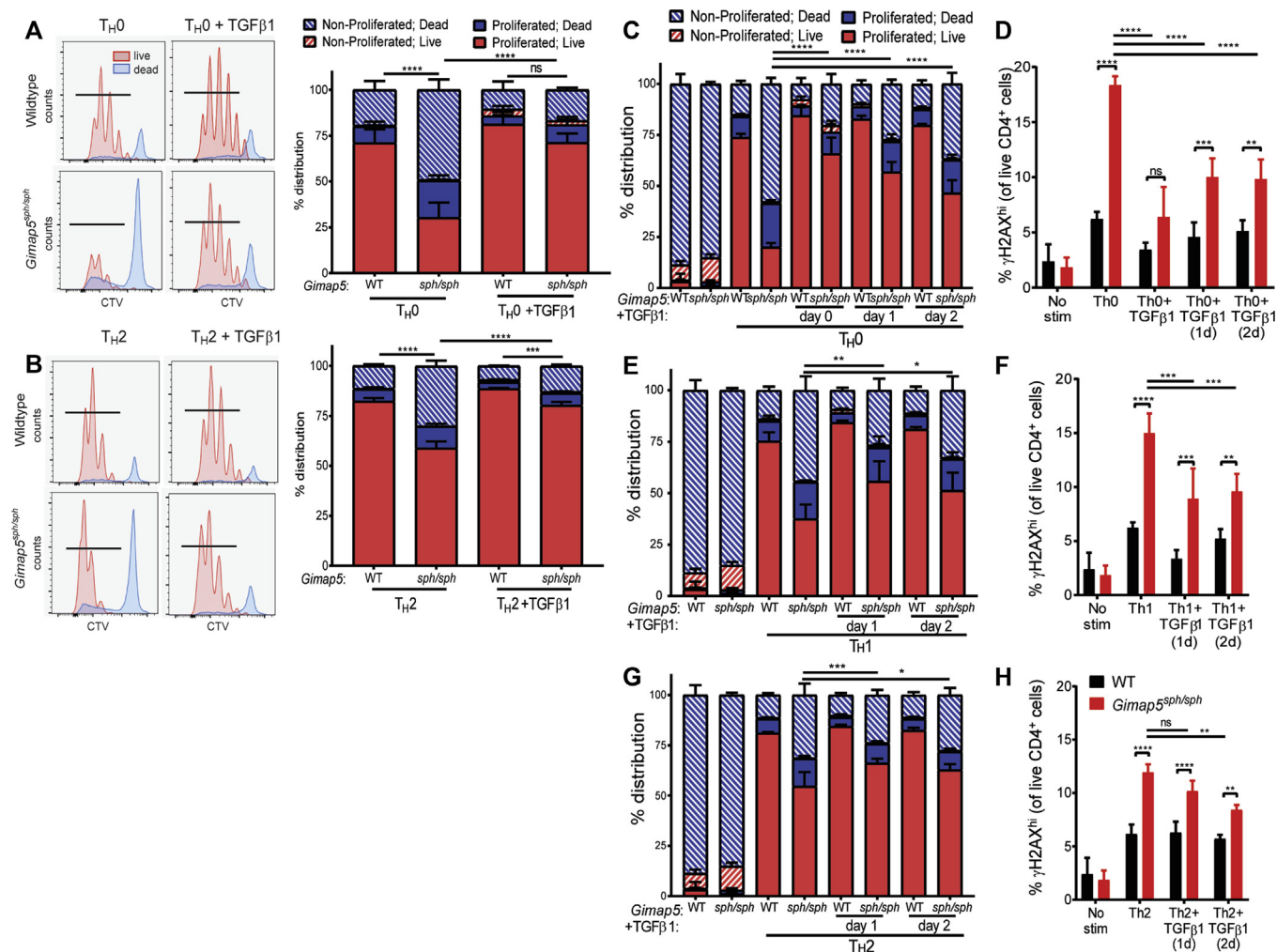


FIG 4. TGF-β1 rescues survival and proliferation of naive *Gimap5*^{sph/sph} CD4⁺ T cells. **A** and **B**, Proliferation and survival of naive WT and *Gimap5*^{sph/sph} CD4⁺ T cells after 3 days of stimulation in Th0-polarizing (Fig 4, A) or Th2-polarizing (Fig 4, B) conditions with or without 2 ng/mL TGF-β1. Blue histograms represent dead cells; red histograms show live cells. **C-H**, Survival, proliferation, and γH2AX expression of naive CD4⁺ T cells stimulated in Th0-polarizing (Fig 4, C and D), Th1-polarizing (Fig 4, E and F), or Th2-polarizing (Fig 4, G and H) conditions. At 1 or 2 days after initial stimulation, 2 ng/mL TGF-β1 was added to the indicated culture. Data represent mean values ± SDs (n = 4). Statistical significance is determined by means of ANOVA, followed by the Sidak multiple comparisons test. *P < .05, **P < .01, ***P < .001, and ****P < .0001. ns, Not significant.

Total lung infiltrating cells from the upper right lobe for each mouse were quantified, restimulated with PMA and ionomycin, and evaluated for cytokine production to determine whether exacerbated AHR and eosinophil/macrophage infiltration correlated with increased development of pathogenic CD4⁺ T cells. Lungs from HDM-sensitized *Gimap5*^{sph/sph} mice had significantly more inflammatory infiltrate (Fig 5, E) and, importantly, exhibited a 4-fold increase in infiltrating CD4⁺ T cells compared with WT mice (Fig 5, F). The latter involved a marked increase in numbers of CD4⁺ T cells producing IL-13 or IL-17 (Fig 5, G and H) and importantly also included a significant increase in numbers of pathogenic IL-13⁺IL-17A⁺ CD4⁺ T cells (Fig 5, I), which have been associated with severe immune pathology/disease. Similarly, lung-draining mediastinal lymph nodes of *Gimap5*^{sph/sph} mice displayed an increase in numbers of IL-13⁺, IL-17A⁺, and IL-13⁺IL-

17A⁺ CD4⁺ T cells (see Fig E4 in this article's Online Repository at www.jacionline.org). Furthermore, HDM sensitization resulted in distortion of normal lung architecture, increased airway mucus production, marked edema, and lymphocytic infiltration in the interstitial space representative of perivascular inflammation (Fig 5, K and L). Together, these data show that LOF mutations in *Gimap5* exacerbate the asthma phenotype, which is associated with a significant expansion of pathogenic Th2/Th17 cells.

Polarization defects in *Gimap5*-deficient CD4⁺ T cells are cell intrinsic

In addition to CD4⁺ T cells, *Gimap5* is expressed in innate lymphoid cells (ILCs), and our previous studies indicate that natural killer cell development/survival is markedly affected in

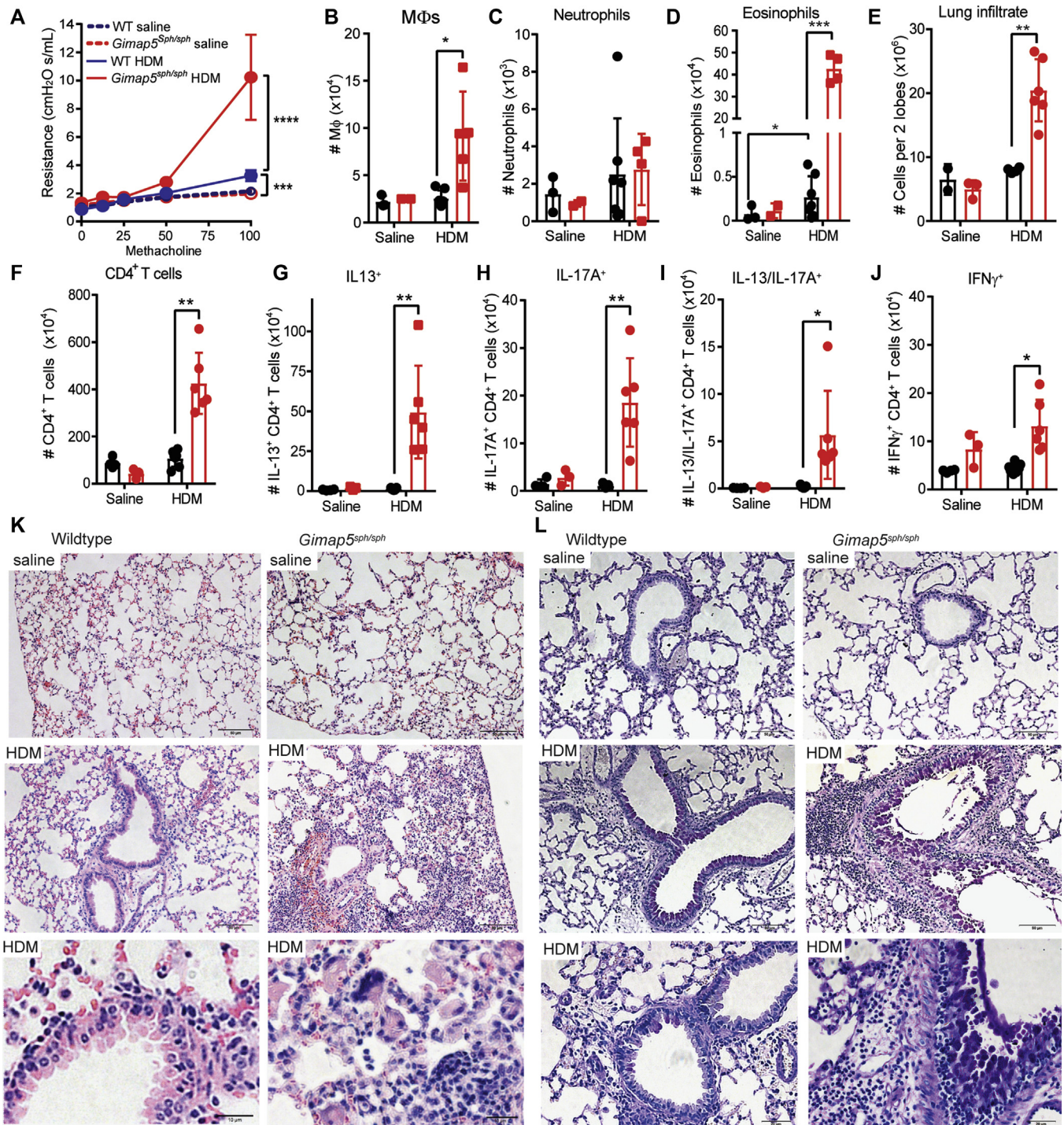


FIG 5. *Gimap5^{sph/sph}* mice exhibit increased AHR, CD4⁺ T-cell polarization, and airway pathology on HDM sensitization. C57BL/6 WT (black bars) and *Gimap5^{sph/sph}* (red bars) mice were administered 25 μ g of HDM 9 times intratracheally over 3 weeks. **A**, AHR in response to methacholine challenge 1 day after the last HDM challenge (saline, *n* = 3; HDM, *n* = 5-6). **B-D**, Numbers of macrophages (MΦs), neutrophils, and eosinophils in BAL fluid (saline, *n* = 3; HDM, *n* = 4-7). **E** and **F**, Numbers of total lung-infiltrating cells (Fig 5, **E**) and CD4⁺ T cells (in 2 lobes; Fig 5, **F**). **G-J**, Percentage of lung-infiltrating CD4⁺ CD3⁺ T cells expressing IL-13, IL-17A, IL-13 and IL-17A, or IFN- γ on restimulation with PMA/ionomycin for 6 hours (saline, *n* = 3; HDM, *n* = 6-7). **K** and **L**, Representative hematoxylin and eosin-stained (Fig 5, **K**) and periodic acid-Schiff-stained (Fig 5, **L**) images of lung tissue from WT and *Gimap5^{sph/sph}* mice given saline or HDM. Data represent mean values $\pm$ SDs. Statistical significance is determined by means of ANOVA, followed by the Sidak multiple comparisons test. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

Gimap5-deficient mice.^{12,19} Given the critical role for type 2 ILCs and type 3 ILCs in CD4⁺ T-cell differentiation and asthma,⁴⁰⁻⁴⁵ we next asked whether the observed CD4⁺ T-cell defects were cell intrinsic or whether other cell types might contribute to the impaired polarization of CD4⁺ T cells. To do so, we generated the *Gimap5*^{fl/fl} mouse by using CRISPR/Cas9 on a C57BL/6 background. *Loxp* sites were placed on flanking exon 2 of *Gimap5* (see Fig E1), and subsequent *Gimap5*^{fl/fl} mice were crossed to *Cd4*^{cre-ert2} mice, allowing the tamoxifen-inducible deletion of *Gimap5* in CD4⁺ T cells (conditional knockout [cKO]). Effective ablation of *Gimap5* expression in CD4⁺ T cells was achieved with the administration of tamoxifen in chow (Fig 6, A).

Similar to CD4⁺ T cells isolated from the *Gimap5*^{sph/sph} mouse, *Gimap5*^{fl/fl}*Cd4*^{cre-ert2} naive CD4⁺ T cells demonstrated impaired survival and proliferation in T_H0-, T_H1-, and T_H2-polarizing conditions compared with that seen in WT control mice. Furthermore, no significant difference in survival and proliferation between WT and cKO CD4⁺ T cells was observed in T_H17-polarizing conditions (Fig 6, B and C). Together, these results correlated with DNA damage observed in cycling CD4⁺ T cells with cKO CD4⁺ T cells displaying increased γ H2AX levels compared with WT CD4⁺ T cells stimulated in T_H0-, T_H1-, T_H2-, and iTreg-polarizing conditions. Similar to *Gimap5*^{sph/sph} CD4⁺ T cells, no difference in survival/proliferation was observed in T_H17-polarizing conditions. These data confirm that the impaired survival and increased DNA damage in stimulated *Gimap5*-deficient CD4⁺ T cells are cell intrinsic.

Loss of *Gimap5* in CD4⁺ T cells is sufficient to drive exacerbated AHR and pathology

Because pathogenic CD4⁺ T cells are one of the primary drivers of HDM-induced lung pathology, we investigated whether *Gimap5* deficiency in CD4⁺ T cells alone was sufficient to drive more severe disease in the HDM model of allergic asthma. To do so, 1 week after starting the tamoxifen chow, allergic airway disease was induced in WT and *Gimap5*^{fl/fl}*Cd4*^{cre-ert2} mice as above. Comparable with *Gimap5*^{sph/sph} mice, conditional deletion of *Gimap5* in CD4⁺ T cells was sufficient to drive increased AHR in HDM-sensitized mice (Fig 7, A). Although exacerbated AHR was not specifically associated with changes in the numbers of macrophages and neutrophils in BAL fluid, the number of infiltrating eosinophils trended upward in HDM-sensitized *Gimap5*^{fl/fl}*Cd4*^{cre-ert2} mice compared with WT mice (Fig 7, B-D). Interestingly, although the numbers of lung-infiltrating CD4⁺ T cells in HDM-sensitized *Gimap5*^{fl/fl}*Cd4*^{cre-ert2} mice were similar or even slightly reduced compared with those in WT mice (Fig 7, E and F), a greater proportion expressed an activated phenotype (CD44^{hi}; Fig 7, G). Furthermore, they exhibited an increased frequency of CD4⁺ T cells producing IL-13, IL-17A, or both IL-17A and IL-13 together (Fig 7, H-J), whereas the frequency of IFN- γ ⁺CD4⁺ T cells was reduced compared with that in WT mice (Fig 7, K). Notably, similar levels of CD4⁺ Treg cells were observed in the lungs of WT and *Gimap5*^{fl/fl}*Cd4*^{cre-ert2} mice, indicating that the development of iTreg cells and/or recruitment is not affected in this model (see Fig E5, A, in this article's Online Repository at www.jacionline.org). B cells were likewise unaffected (Fig E5, B). Similar to the *Gimap5*^{sph/sph} mice, HDM sensitization resulted in edema with perivascular inflammation and increased mucus production in the lungs of cKO mice (Fig 7, L and M). These data reveal that the conditional loss of *Gimap5* in CD4⁺ T cells is

sufficient to drive the development of pathogenic CD4⁺ T cells and exacerbate allergy-like lung disease upon HDM sensitization.

Because we have previously shown that *Gimap5*-deficient Treg cells have impaired suppressive capacity, we next asked whether defective Treg cells were responsible for the development of more severe airway disease. Interestingly, selective ablation of *Gimap5* in Treg cells was insufficient to drive exacerbated airway disease and development of pathogenic CD4⁺ T cells, indicating that loss of *Gimap5* intrinsically promotes the polarization of pathogenic CD4⁺ T cells (see Fig E2).

DISCUSSION

Here we describe a key role for *Gimap5* in the prevention of pathogenic CD4⁺ T-cell development and show that loss of *Gimap5* is associated with an increased risk for allergic lung disease. Specifically, HDM sensitization of *Gimap5*-deficient mice results in severe AHR, eosinophil recruitment to the lung, and an exacerbated T_H2/T_H17 response. The increased airway inflammation was also observed in conditional loss of *Gimap5* in CD4⁺ T cells, demonstrating that these cells are the primary driver of HDM-induced lung pathology. Moreover, even in the absence of HDM sensitization, *Gimap5*^{sph/sph} CD4⁺ T cells progressively adopt an inflammatory phenotype, with T_H17 cells being particularly prominent. Importantly, the abnormal CD4⁺ T-cell polarization profile is also observed in a GIMAP5-deficient patient who exhibited increased development of pathogenic CD4⁺ T cells in the periphery. Thus despite the observed lymphopenia in both *Gimap5*-deficient mice and human subjects, the selective survival of pathogenic T_H17 cells might be a key driver of immune pathology in the setting of allergic airway disease.

Our current findings that loss of GIMAP5 leads to pathogenic CD4⁺ T-cell development might help us understand how the *GIMAP5* polymorphism rs6965571 in the promoter region of *GIMAP5* predisposes to asthma development.²⁵ The rs6965571 SNP is adjacent to a Foxo1 binding site (upstream) and a nuclear factor of activated T cells 1 (NFATc1) binding motif (downstream) and could affect the binding of these transcription factors (TFs) to regulate *GIMAP5* expression. Interestingly, our previous studies identified impaired Foxo1 expression¹⁵ and NFATc1 activity on stimulation of *Gimap5*-deficient CD4⁺ T cells.¹⁴ Thus a potential feed-forward loop exists in which *Gimap5* expression is required for the expression and activity of TFs that in turn regulate the transcriptional expression of *GIMAP5*. Thus the SNP can cause a progressive loss of *GIMAP5* expression after T-cell activation, leading to increased development of T_H2/T_H17 cells that promote immune pathology in the lung. Previously, the presence of T_H2/T_H17 cells has been associated with more severe disease, and adoptive transfer experiments in mice have demonstrated that these cells are more pathogenic than classic T_H2 cells.²⁻⁴ Furthermore, increased levels of IL-17A, either from these double producers or classic T_H17 cells, have been linked to more severe disease and steroid resistance in human patients.⁵⁻¹¹ These increased IL-17A levels can drive increased pathology directly through recruitment of neutrophils, or indirectly by modulating T_H2 pathogenicity by enhancing IL-13/signal transducer and activator of transcription 6 signaling, as shown by Hall et al.⁴⁶ Thus a critical future question to address is to identify the effect of the rs6965571 polymorphism on GIMAP5 expression on T-cell activation and to establish whether patients with this SNP have an increased number of pathogenic (T_H17) CD4⁺ T cells.

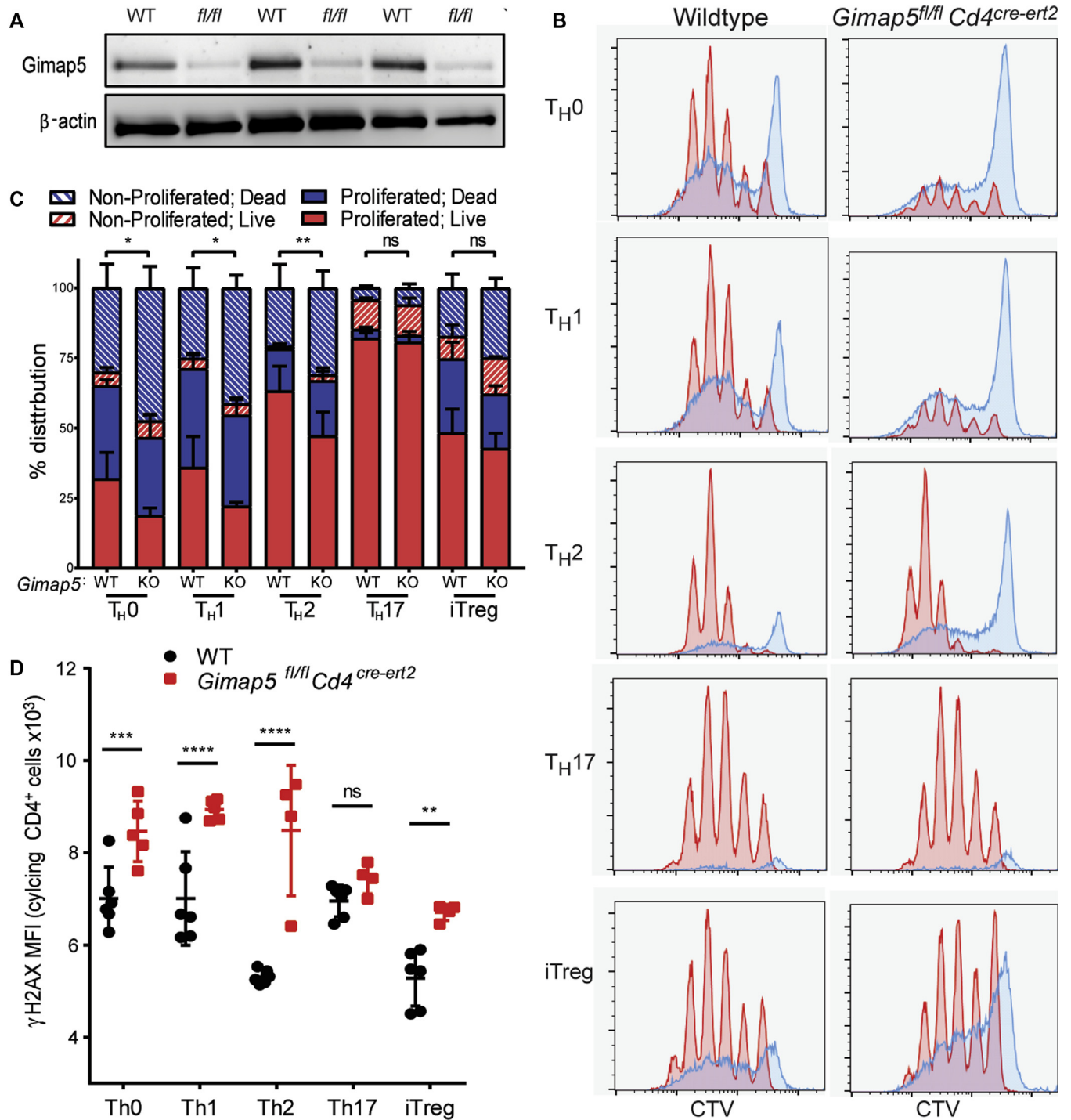


FIG 6. Impaired survival and increased DNA damage associated with *Gimap5* deficiency is CD4⁺ T-cell intrinsic. **A**, Expression of *Gimap5* in CD4⁺ T cells isolated from WT and *Gimap5^{fl/fl} Cd4^{cre-ert2}* mice after tamoxifen-induced deletion of *Gimap5*. Immunoblotting shows 3 independent cKO mice. **B** and **C**, Proliferation and survival of naive CD4⁺ T cells from WT and *Gimap5^{fl/fl} Cd4^{cre-ert2}* mice stimulated for 3 days in non-polarizing conditions (T_H0) or T_H1-, T_H2-, T_H17-, or iTReg-polarizing conditions. Blue histograms represent dead cells; red histograms show live cells. **D**, γH2AX expression in live cycling CD4⁺ T cells after 3 days pf stimulation in T_H0-, T_H1-, T_H2-, T_H17-, or iTReg-polarizing conditions. Data represent mean values ± SDs (n = 4-5). Statistical significance is determined by means of ANOVA, followed by the Sidak multiple comparisons test. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. ns, Not significant.

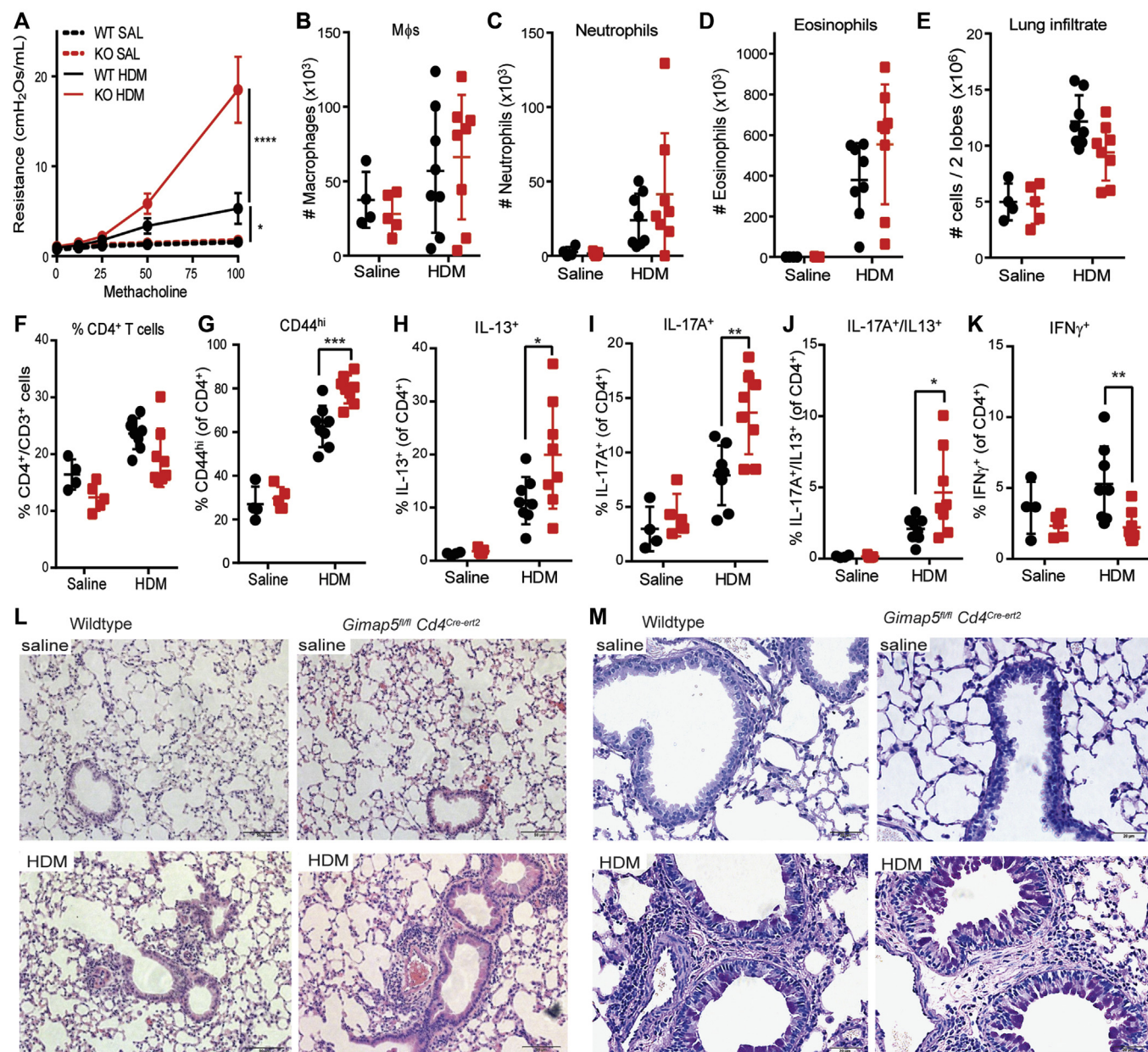


FIG 7. *Gimap5* deficiency in CD4⁺ T cells is sufficient to drive increased AHR, CD4⁺ T-cell polarization, and airway pathology on HDM sensitization. C57BL/6 WT (black data points) and *Gimap5*^{fl/fl} *Cd4*^{Cre-ert2} (red data points) mice were administered 50 μ g of HDM 9 times intratracheally over 3 weeks. **A**, AHR in response to methacholine challenge 1 day after the last HDM challenge (saline, $n = 4-5$; HDM, $n = 7-8$). **B-D**, Number of macrophages (*M* ϕ s), neutrophils, and eosinophils in BAL fluid (saline, $n = 4-5$; HDM, $n = 8$). **E** and **F**, Number of total lung-infiltrating cells (in 2 lobes; Fig 7, **E**) and frequency of CD4⁺CD3⁺ T cells therein (Fig 7, **F**). **G**, Frequency of CD4⁺CD3⁺ T cells expressing an activated (CD44^{hi}) phenotype. **H-K**, Percentage of lung-infiltrating CD4⁺CD3⁺ T cells expressing IL-13, IL-17A, IL-13 and IL-17A, or IFN- γ on restimulation with PMA/ionomycin for 6 hours (saline, $n = 4-5$; HDM, $n = 8$). **L** and **M**, Representative hematoxylin and eosin (Fig 7, **L**) and periodic acid-Schiff (Fig 7, **M**) images of lung tissue from WT and *Gimap5*^{fl/fl} *Cd4*^{Cre-ert2} mice given saline or HDM. Data represent mean values $\pm$ SDs. Statistical significance is determined by means of ANOVA, followed by the Sidak multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

Although DNA damage occurs normally upon mitogenic stimulation in CD4⁺ T cells, a key finding is that the exacerbated DNA damage in cycling *Gimap5*-deficient CD4⁺ T cells and reduced T-cell survival is independent of Fas-signaling or intrinsic apoptotic cascades.¹⁴ Importantly, our previous studies identified a key role for *Gimap5* in regulating glycogen synthase

kinase 3 (GSK3) activity and subsequent control of the DNA damage response during CD4⁺ T-cell proliferation.¹⁴ Here we show that DNA damage was particularly associated with T_H1- and T_H2-polarizing conditions. Interestingly, the presence of TGF- β could limit the extent of DNA damage, even in T_H1- and T_H2-polarizing conditions. Previous studies have shown

that TGF- β signaling can promote DNA damage repair and cell viability by inducing activation of ataxia-telangiectasia mutated (ATM),^{47,48} suggesting that TGF- β signaling can overcome the exacerbated DNA damage observed in activated Gimap5-deficient CD4⁺ T cells. Moreover, we show that initiation of TGF- β signaling even 2 days after initial T-cell receptor stimulation is sufficient to reduce cell death and DNA damage *in vitro* in Gimap5-deficient CD4⁺ T cells. *In vivo* this would translate to improved survival of newly activated CD4⁺ T cells encountering TGF- β in the lung or other TGF- β -rich tissues. We posit that the ability of TGF- β to overcome the survival defects associated with Gimap5 deficiency could affect the CD4⁺ T-cell compartment by promoting the selective survival of T_H17 cells and other pathogenic CD4⁺ T cells infiltrating the TGF- β -rich asthmatic lung, where they drive further immune pathology.

Somewhat paradoxically, Gimap5-deficient mice do not induce iTreg cells *in vivo*, and retained Treg cells have reduced suppressive capacity.^{14,15} It remains unclear why TGF- β rescues T_H17 CD4⁺ T-cell development but not iTreg polarization and function, although our previous data suggest that the uncontrolled activity of GSK3 β in the absence of Gimap5 might be critical to this process. Several independent studies demonstrate that GSK3 β activity promotes T_H17 polarization while impairing iTreg cell activity, providing one possible mechanism by which T_H17 polarization is favored in the absence of Gimap5.⁴⁹⁻⁵⁵ Furthermore, Foxo family members, which are poorly expressed in Gimap5-deficient CD4⁺ T cells,¹⁵ are key TFs in Treg cell development and function.^{56,57} Impaired expression of these TFs is predicted to be a critical factor in the inability of *Gimap5*^{sph/sph} mice to develop iTreg cells. This dysbalance between pathogenic CD4⁺ T cells and functional Treg cells is one of the primary driving factors behind the immune pathologies associated with Gimap5 deficiency. Unfortunately, the inability of TGF- β to rescue iTreg cell function, its promotion of the development of pathogenic T_H17 CD4⁺ T cells, and its critical role in the resolution of inflammation make it an impractical target in clinical applications. However, therapeutic targeting of other links in the GIMAP5 pathway, such as GSK3 β , might offer more promise in clinical options for patients with GIMAP5-associated asthma.

We thank G. Butcher for graciously providing the MAC421 α Gimap5 antibody.

Key messages

- *GIMAP5* LOF mutations in mice and human subjects result in increased T_H2/T_H17 polarization *in vivo*.
- Gimap5-deficient CD4⁺ T cells exhibit increased DNA damage and reduced survival on activation that can be overcome by TGF- β .
- Gimap5-deficient mice have more severe allergic airway disease on HDM sensitization.

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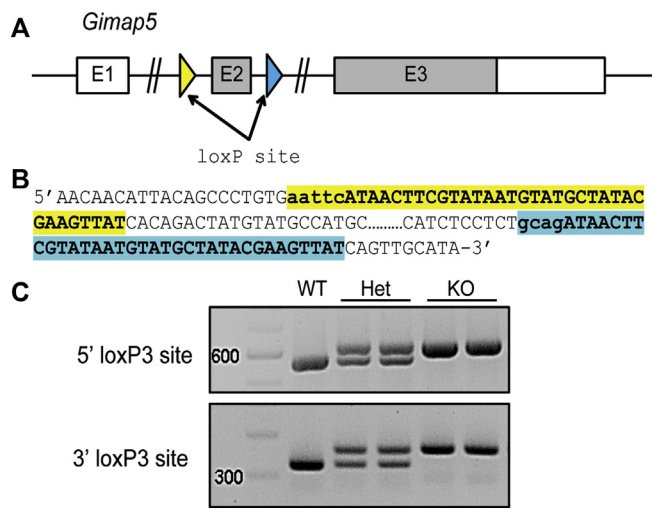


FIG E1. Generation of the *Gimap5*^{fl/fl} allele. **A**, Schematic of the *Gimap5*^{fl/fl} allele. *LoxP* sites flank exon 2. **B**, Sequence of the *Gimap5*^{fl/fl} allele with the *LoxP* inserts highlighted. **C**, Gel of the PCR product determining the presence/absence of the *Gimap5*^{fl/fl} allele. Primers are listed in the Methods section.

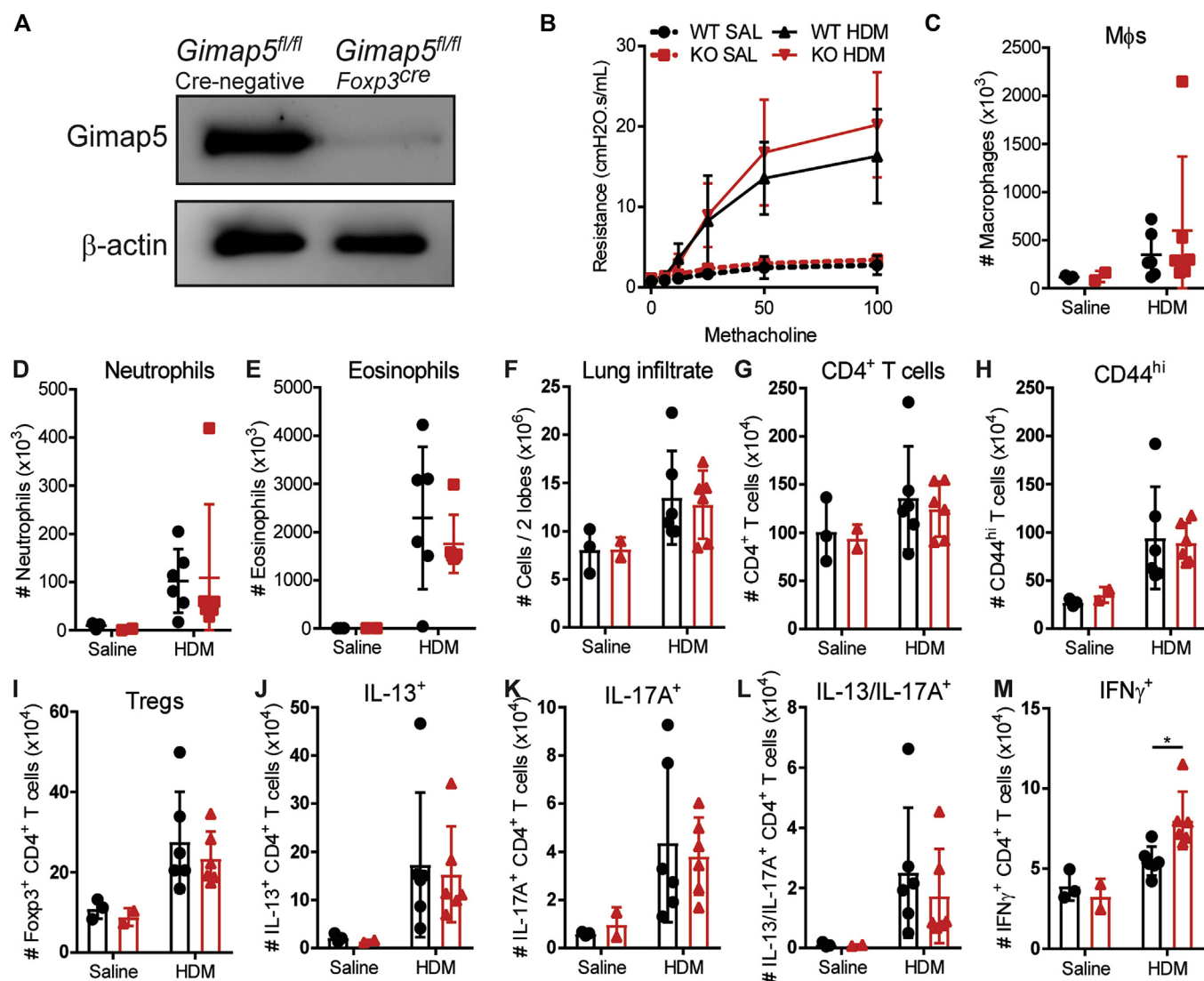


FIG E2. Selective ablation of *Gimap5* in *Foxp3*⁺ Treg cells does not affect HDM-induced airway disease. C57BL/6 WT (black data points) and *Gimap5^{fl/fl}* *Foxp3^{cre}* (red data points) mice were administered 50 μ g of HDM 9 times intratracheally over 3 weeks. **A**, Expression of *Gimap5* in CD4⁺CD25^{hi} T cells isolated from WT and *Gimap5^{fl/fl}* *Foxp3^{cre}* mice. **B**, AHR in response to methacholine challenge 1 day after the last HDM challenge. **C–E**, Number of macrophages (*M* ϕ s), neutrophils, and eosinophils in BAL fluid. **F** and **G**, Number of total lung-infiltrating cells (Fig E2, **F**) and CD4⁺CD3⁺ T cells (Fig E2, **G**) in 2 lobes. **H** and **I**, Frequency of CD4⁺CD3⁺ T cells expressing an activated (CD44^{hi}) phenotype (Fig E2, **H**) and numbers of Foxp3⁺ Treg cells (Fig E2, **I**). **J–M**, Percentage of lung-infiltrating CD4⁺CD3⁺ T cells expressing IL-13, IL-17A, IL-13 and IL-17A, or IFN- γ on restimulation with PMA/ionomycin for 6 hours. Data represent mean values $\pm$ SDs (saline, *n* = 2–3; HDM, *n* = 6). Statistical significance is determined by means of ANOVA, followed by the Sidak multiple comparisons test. **P* < .05.

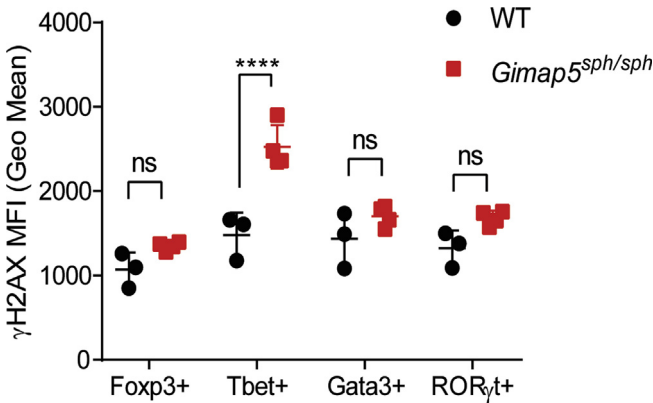


FIG E3. Gimap5-deficient T_H1 cells exhibit increased DNA damage *in vivo*. **A**, γH2AX levels in splenic CD4⁺ T cells expressing Foxp3 (Treg cells), T-bet (T_H1 cells), Gata-3 (T_H2 cells), and RORγt (T_H17 cells). Data represent mean values ± SDs (n = 3-4). Statistical significance is determined by using the Student 2-tailed test. ****P < .0001. ns, Not significant.

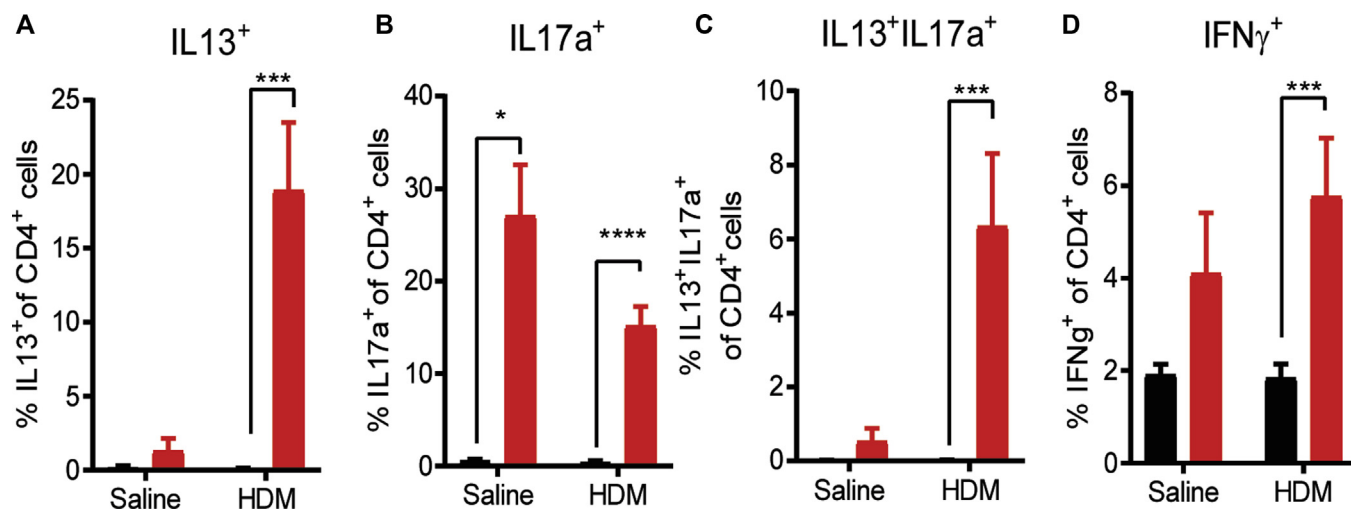


FIG E4. Increased polarization of *Gimap5^{sp/sph}* CD4⁺ T cells in the mediastinal lymph nodes in saline- and HDM-treated mice. Frequency of CD4⁺ T cells from the mediastinal lymph nodes expressing IL-13, IL-17a, and IFN-γ after restimulation with PMA/ionomycin. *Black bars* describe WT mice; *red bars* describe *Gimap5^{sp/sph}* mice. Data represent mean values ± SDs (saline, n = 3; HDM, n = 6-7). Statistical significance is determined by means of ANOVA, followed by the Sidak multiple comparisons test. *P < .05, ***P < .001, and ****P < .0001.

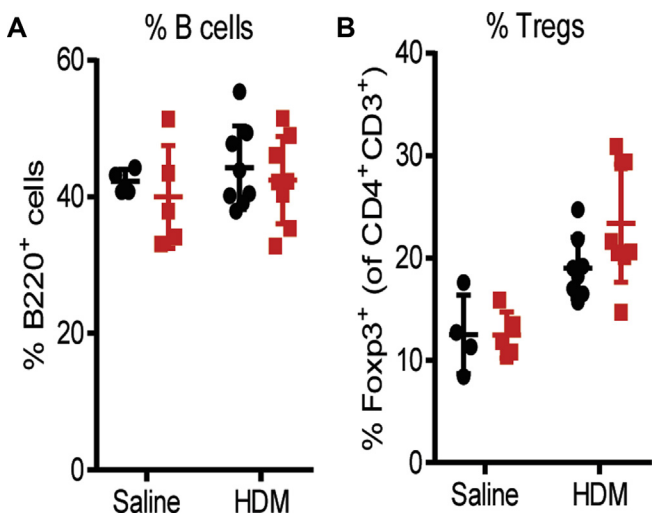


FIG E5. Frequencies of Treg and B cells are unaffected in *Gimap5^{fl/fl} Cd4^{cre-ert2}* mice. **A,** Frequency of Foxp3⁺ CD4⁺ T cells in the lungs of saline- and HDM-sensitized mice. **B,** Frequency of lung-infiltrating B cells. *Black data points* describe WT mice; *red data points* describe *Gimap5^{sph/sph}* mice. Data represent means $\pm$ SDs (saline, n = 4-5; HDM, n = 8).

TABLE E1. Polarizing conditions for T_H cell differentiation
in vitro

Polarizing condition	Reagents (antibodies/cytokines)
T _H 0	1 μg/mL αCD3; 2 μg/mL αCD28
T _H 1	T _H 0 + 1 μg/mL αIL-4; 10 ng/mL mIL-12
T _H 2	T _H 0 + 10 μg/mL αIFN-γ; 20 ng/mL mIL-4
iTreg	T _H 0 + 1 μg/mL αIL-4; 10 μg/mL αIFN-γ; 2 ng/mL TGF-β1; 100 U/mL hIL-2
T _H 17 (1)	T _H 0 + 1 μg/mL αIL-4; 10 μg/mL αIFN-γ; 2 ng/mL TGF-β1; 20 ng/mL mIL-6; 10 ng/mL mIL-1β
T _H 17 (2)	T _H 0 + 1 μg/mL αIL-4; 10 μg/mL αIFN-γ; 2 ng/mL TGF-β1; 20 ng/mL mIL-6; 20 ng/mL mIL-23
T _H 17 (3)	T _H 0 + 1 μg/mL αIL-4; 10 μg/mL αIFN-γ; 2 ng/mL TGF-β3; 20 ng/mL mIL-6; 10 ng/mL mIL-1β; 20 ng/mL mIL-23
T _H 17 (4)	T _H 0 + 1 μg/mL αIL-4; 10 μg/mL αIFN-γ; 10 μg/mL αTGF-β; 20 ng/mL mIL-6; 10 ng/mL mIL-1β; 20 ng/mL mIL-23