# CONTINUED FASCINATION – A TRIBUTE TO A GIANT IN IMMUNOLOGY, DR. WILLIAM E. PAUL

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# CONTINUED FASCINATION – A TRIBUTE TO A GIANT IN IMMUNOLOGY, DR. WILLIAM E. PAUL

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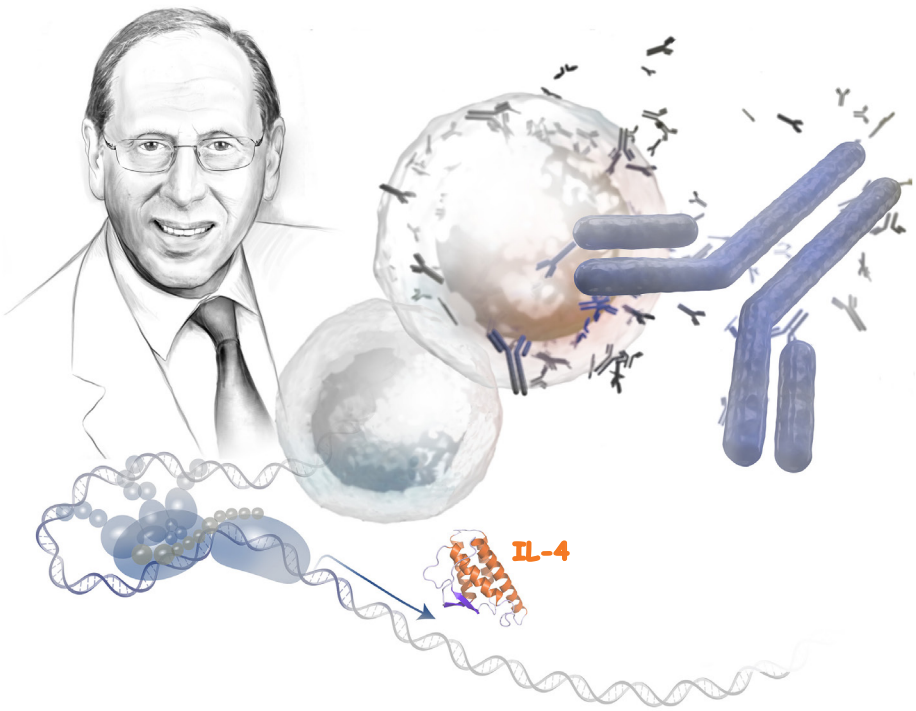


A portrait of Dr. Paul and his fascination; courtesy of Julia Fekecs and Darryl Leja of NHGRI.

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Dr. William E. Paul (1936–2015) was the leader of the National Institutes of Health (NIH) immunology community and his career is without parallel in the field of immunology. He was the Chief of the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases (NIAID), from 1970 at the age of 34 until his death. His groundbreaking contributions to the field of immunology, including the discovery of interleukin (IL)-4, led to more than 600 publications over half a century. He also played an important role in the establishment of the NIH Vaccine Research Center while he was the Director of the NIH Office of AIDS Research. Furthermore, Dr. Paul was a shining icon and an international giant of contemporary immunology. He was a genius and a living encyclopedia of immunology: the author of the textbook *Fundamental Immunology* since its inception to the 7th edition in 2013; and the editor of the *Annual Review of Immunology* from its inaugural issue in 1983 until 2011. In his last book *Immunity*, he discussed the three laws of immunology: universality, tolerance and appropriateness. These capture the essence of Dr. Paul

as well as the field. Dr. Paul had an enormous impact on the research career of his trainees, many of whom became leaders in the field of immunology, including Drs. Charles Janeway, Ronald Schwartz, Laurie Glimcher and Mark Davis. Dr. Paul was an intelligent, generous, humble but optimistic man. He was also an inspirational and thoughtful leader, colleague and friend; he inspired and encouraged people around him in every possible way. As his trainees and/or colleagues, we miss him greatly and dedicate this special Research Topic to his memory. We thank all the authors who participated in this collection as well as other colleagues and friends of Dr. Paul's who have supported us in a series of events after Dr. Paul's passing. Finally, we would like to thank the *Frontiers in Immunology* for providing such a wonderful platform for remembering Dr. Paul's remarkable life.

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EDITORIAL
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# Editorial: Continued Fascination–A Tribute to a Giant in Immunology, Dr. William E. Paul

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## Editorial on the Research Topic

## Continued Fascination–A Tribute to a Giant in Immunology, Dr. William E. Paul

This issue honors the memory of Dr. William E. Paul, a towering figure in immunology for decades (1). Working at the Laboratory of Immunology within the NIH-NIAID for over 45 years, Bill's scientific legacy was extraordinary, matched only by the legacy of mentoring prowess (2, 3). In these pages are a series of 16 articles (15 reviews and 1 research article), most of which are authored by Bill's former trainees. It is noteworthy that Bill would often refer to those in his lab as colleagues, no matter how junior the lab member was. While this title may have felt not fitting to the trainee, there is little doubt that Bill had both a respect for those who worked for him and an expectation that one day they would have achievements worthy of making them his colleague. This gesture may well have served as one of many motivating factors which led to the extraordinary array of individuals who are Bill's legacy.

The articles in this issue cover the vast interests and expertise that germinated within Bill's lab and in the Laboratory of Immunology, and now flower throughout the scientific and medical world—validation of Bill's prescience in the title he chose for his trainees. They include the gamut of basic, translational and clinical findings covering topics which were close to home for Bill, such as the effects and regulation of IL-4 and IL-13 in various cell types (Prout et al.; Keegan et al.; Yoshimoto; Junttila), the regulation of T-cell differentiation (Zhu; Milner), T cell homeostasis (Min), mast cell biology (Brown; Caslin et al.; Huang et al.), and B cell activation (DeFranco), to those that go beyond as individual trainees followed their own unique paths (Nakanishi; Snapper). This issue also contains many important research topics that are the focuses of some independent groups (Zhu; Shevach; Kanellopoulou and Muljo; Natarajan et al.) within the Laboratory of Immunology, where Bill had served as the Lab Chief.

Below, we also include an introduction to this issue by Bill's beloved wife Marilyn, which provides all of us a more complete memory of Bill, whose endless fascination with, and contributions to the world went well-beyond science. Hopefully these articles and Mrs. Paul's personal reflections will be enlightening to the reader as the state of the art in immunology and beyond, but also provide a reminder of the enormous impact Bill has had in shaping the art, and the artists and whose work is presented here. There is no doubt that Bill's endless fascination continues.

Bill was lucky. He knew from childhood that he wanted to be a scientist. No ambivalence, no doubts. His parents wanted him to be a doctor but he wanted to be a scientist so he became both. He was lucky that he lived in Brooklyn, New York where Brooklyn College was free. His college sport was fencing. After college, he attended medical school at SUNY Downstate Medical School, also in Brooklyn. As a student, Bill lived at home with his parents. He and I married during his third year of medical school and created the home that we would share for the next 57 years. I felt so lucky that he chose me.

Although he always studied hard, he found joy in learning. He loved learning and was learned and well-read in many subjects, humanities as well as science.

Once he discovered immunology, he became devoted to it and excited by its power and potential. Immunology became his scientific home. He was proud to be a member of the generation of scientists that opened the doors of immunology to the scientific and medical academic world.

Bill loved working at the National Institutes of Health. Over the years, he had many attractive opportunities to move elsewhere. We talked it over and he stayed at NIH. He thought NIH was the best place to do basic science. He thought NIH was the best place to mentor the next generation of scientists. Bill was ever concerned that the scientific *enterprise*, his word not mine, continue.

He reveled in scientific success, his own and the achievements of others. He would come home excited and try to explain to me the importance of a recent discovery whether it was from his laboratory or elsewhere. He took special satisfaction in seeing his post docs and fellows succeed, make contributions and find jobs heading influential medical research institutions.

He was sought after as a consultant by many scientific research organizations and he thought it was important to offer his expertise to guide their programs. He wanted them to spend their money wisely. They were always surprised that he did so much work for them without being able to accept compensation. I can still picture in my mind the reams of paper he brought home to read each night until digital computer technology literally lightened the load.

In 1994, at the peak of the AIDS epidemic he took on the responsibility of becoming the Director of the Office of AIDS Research. We both understood that this was a scary time for AIDS patients and AIDS workers as well. There was a huge very ill population out there. They were scared, felt threatened and wanted a drug fast. They were angry and impatient. While working in this crisis Bill realized that more rigorous attention should be paid to vaccine research. He proposed the development of a vaccine research center for the NIH and was part of the delegation of scientists who went to the White House to convince President Bill Clinton of its importance. Clinton was persuaded. The Vaccine Research Center (VRC) opened for business in 2001. One could think of Bill as the Father of the VRC as it was his idea. I was told recently that the Building 40 of VRC went up faster than most other structures on the campus, when I attended the ceremony of dedicating the Conference Room at the VRC to Bill's memory.

Every day that he went to work was a joyful day. Every evening that he worked at home was a joyful evening. At the end of the day, he told me he quit only when he realized that he had to read something three times. He took that as a signal he was tired and should stop and have some ice cream, well-deserved ice cream.

As I stated in the beginning, Bill was lucky to find a gratifying career interest early in his life. Our sons and I were lucky too. We had the pleasure of living with a man who was so happy in his life's work. I can only hope that his life will serve as a role model for our young grandchildren. Wouldn't that be wonderful?

## AUTHOR CONTRIBUTIONS

JM and JZ wrote the first part. MP wrote the second part.

## ACKNOWLEDGMENTS

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# IL-4 Is a Key Requirement for IL-4- and IL-4/IL-13-Expressing CD4 Th2 Subsets in Lung and Skin

*Melanie Sarah Prout, Ryan L. Kyle†, Franca Ronchese and Graham Le Gros**

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Although IL-4 is long associated with CD4 Th2 immune responses, its role in Th2 subset development in non-lymphoid tissues is less clear. We sought to better define IL-4's role in CD4 Th2 responses by using transgenic mice that express a dual IL-4 AmCyan/IL-13 DsRed (IL-4AC/IL-13DR) fluorescent reporter on an IL-4-sufficient or IL-4-deficient background. Using primary Th2 immune response models against house dust mite or *Nippostrongylus brasiliensis* (*Nb*) allergens, we examined the requirement for IL-4 by each of the defined Th2 subsets in the antigen draining lymph node, skin, and lung tissues. In the lymph node, a CXCR5$^{+}$PD-1$^{+}$ T follicular helper (Tfh) and a CXCR5$^{lo}$PD-1$^{lo}$ Th2 subset could be detected that expressed only IL-4AC but no IL-13DR. The number of IL-4AC$^{+}$ Tfh cells was not affected by IL-4 deficiency whereas the number of IL-4AC$^{+}$ Th2 cells was significantly reduced. In the non-lymphoid dermal or lung tissues of allergen primed or *Nb*-infected mice, three strikingly distinct T cell subsets could be detected that were IL-4AC, or IL-4AC/IL-13DR, or IL-13DR CD4. The IL-4- and IL-4/IL-13-expressing subsets were significantly reduced in IL-4-deficient mice, while the numbers of IL-13-expressing CD4 T cells were not affected by IL-4 deficiency indicating that other factors can play a role in directing the development of this Th2 subtype. Taken together, these data indicate that the appearance of IL-4-expressing Tfh cells in the lymph node is not dependent on IL-4 while the appearance of IL-4-expressing Th2 subsets in the lymph node and IL-4, IL-4/IL-13-expressing Th2 subsets in skin and lung tissues of antigen primed mice is significantly IL-4 dependent.

**Keywords: Th2, IL-4, IL-13, skin, lymph node, lung, non-lymphoid tissues**

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## INTRODUCTION

The cytokine IL-4 is understood to be key to the development of type 2 immune responses that underlie allergic disease pathologies and immunity to parasites; however, the specific role IL-4 plays in CD4 T cell differentiation is less clear. Early investigations using *in vitro* culture systems revealed the dominant role that IL-4 plays in driving and shaping CD4 Th2 subset differentiation toward expression of the canonical type 2 cytokines IL-4, IL-5, and IL-13 (1, 2). Although *in vivo* studies have indicated a role for IL-4 directing Th2 development in the lymph node (3, 4), further studies revealed a more subtle role for IL-4 with IL-4-expressing Th2 cells appearing in the draining lymph nodes of immunized mice seemingly independent of IL-4 or STAT6 signaling (5, 6). Other studies have shown that the level of TCR activation can play a role in Th2 differentiation (7), while recent studies even support the view that Th2 development occurs in the tissues and is fully regulated by tissue-specific

checkpoints (8). In addition, TSLP elicited basophils have been shown to promote epicutaneous sensitization to food antigens and subsequent IgE mediated food allergy through IL-4 (9).

We took the opportunity to clarify the role of IL-4 in *in vivo* CD4 Th2 subset differentiation by asking whether certain specific Th2 subsets are more sensitive to the influence of IL-4 and whether IL-4-is required for Th2 subset development at non-lymphoid tissue sites such as the skin and lung. For the *in vivo* immune response studies, we used our recently developed antigen priming ear model (10) to quantitatively analyze the character, kinetics and magnitude of the IL-4- and IL-13-producing Th2 subsets that appear in the ear and ear draining lymph node following allergen priming (11, 12). To examine the appearance Th2 subsets in the lung, we used *Nippostrongylus brasiliensis* infection model which involves a lung migration stage in its infection cycle (13). In following the appearance of IL-4- and IL-13-expressing Th2 subsets, we were concerned to reduce artifact and bias inherent in restimulation and intracellular cytokine staining techniques and previously reported reporter IL-4 knockout mice (14–16). Therefore, we used the validated sensitivity of the recently developed *Il4* and *Il13* transcriptional reporter 4C13R mice (17) to investigate the appearance of IL-4-AmCyan (IL-4AC)- and IL-13-DsRed (IL-13DR)-expressing $CD4^+$ T cells arising in both the lymphoid and non-lymphoid tissues responding to house dust mite (HDM) or *Nippostrongylus brasiliensis* (*Nb*) allergens in either an IL-4-sufficient or -deficient environment. The *Il4*AC/*Il13*DR reporter BAC transgene construct is independent of the endogenous *Il4*/*Il13* locus in the mouse and appears able to faithfully report the commitment of CD4 T cells to the expression of the canonical type 2 cytokine gene expression pattern (17–19) under the appropriate tissue culture and relevant *in vivo* immunization protocols without affecting normal type 2 immune effector functions (20). We were able to detect both IL-4-expressing Tfh and Th2 cells in the draining lymph nodes of HDM challenged mice and while the small number of IL-4AC Th2 cells was significantly reduced by removal of IL-4, the Tfh cells were independent of the need for IL-4. Strikingly, analysis of the CD4 T cells that migrated to the skin 7 days after the allergen challenge revealed three functionally distinct Th2 subsets that could be defined by their cytokine expression patterns, IL-4AC, IL-4AC/IL-13DR, and IL-13DR only. The appearance of the IL-4AC- and IL-4AC/IL-13DR-expressing Th2 cell subsets was highly dependent on IL-4 while the IL-13DR Th2 subset was not affected by the IL-4-deficient background. Taken together, our findings reveal the fundamental role that IL-4 plays in the development of functionally diverse effector Th2 subsets in tissues and lymph node. We also identify a novel IL-13-producing $CD4^+$ Th2 subset that appears in the skin following allergen challenge and does not require IL-4 for expression of IL-13.

# MATERIALS AND METHODS

## Mice

4C13R (17) reporter mice were bred and maintained on a C57BL/6 background in the Malaghan Institute of Medical Research Biomedical Research Unit. The *Il4*AmCyan/*Il13*DsRed construct does not interfere with normal immune function with similar levels of T cells, B cell, immunoglobulin, and inflammatory cells being induced by immunization compared with wild-type C57BL/6 mice. To generate IL-4-deficient reporter strains, 4C13R mice were crossed with IL-$4^{G4/G4}$ mice (6) to generate 4C13R × IL-$4^{G4/+}$ (IL-$4^{+/-}$) mice and 4C13R × IL-$4^{G4/+}$ mice were crossed with IL-$4^{G4/G4}$ mice to generate 4C13R × IL-$4^{G4/G4}$ (IL-$4^{-/-}$) mice. All animal procedures were approved by the Victoria University of Wellington Animal Ethics committee and performed in accordance with institutional guidelines.

## Ear Immunizations

Mice were anesthetized using xylazine and ketamine (Phoenix, New Zealand). 30 µl of a solution containing 200 µg HDM (Greer Laboratories, Lenoir, NC, USA) or 600 dead L3 *Nb* was injected into the ear pinnae as described (10).

## *Nb* Infection

Mice were inoculated with 550 L3 *Nb* larvae by s.c. injection.

## Cell Isolation

All tissues were isolated 7 days posttreatment. Auricular draining lymph nodes were pressed through 70-µm cell strainers into complete media [IMDM (GIBCO) supplemented with 5% FBS (Sigma), 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen), and 55 µM β-mercaptoethanol (Invitrogen)] to make a single-cell suspension. Dorsal and ventral ear sheets were separated and minced into very fine pieces using scissors. Each ear was digested by incubating for 30 min at 37°C with shaking, in ear digestion mix [IMDM (GIBCO) containing 1.2 mg/ml Collagenase IV (Sigma) and 120 µg/ml DNAse I (Roche)]. Solution was pipetted up and down and passed through 70-µm cell strainers into Ear wash buffer [PBS containing 1% BSA (Sigma), 5 mM EDTA (Invitrogen), and 120 µg/ml DNAse I (Roche)]. Cells were washed once more in Ear wash buffer before resuspension in cIMDM. Bronchoalveolar lavage (BAL) was performed by cannulation of mice and washing airways three times with PBS. Lungs were finely minced and digested in lung digestion mix [IMDM (GIBCO) containing 2.4 mg/ml Collagenase I (GIBCO) and 120 µg/ml DNAse I (Roche)] for 1 h at 37°C. Live cell counts performed using a hemocytometer and trypan blue (Invitrogen) exclusion.

## *In Vitro* Restimulation

Day 7 ear lymph node cells ($1 \times 10^6$/well) were cultured for 19 h on plates coated with 1 µg/ml anti-CD3 (145-2C11) with cIMDM, 100 U/ml rIL-2, and 1/50 dilution of anti-CD28 (37.51) supernatant corresponding to 5 µg/ml.

## FACS Analysis

Cells were resuspended in a buffer containing 0.01% $NaN_3$ (Sigma), 2% FBS, and 2 mM EDTA (Life Technologies) in PBS then incubated with anti-CD16/32 antibody (clone 2.4G2) before staining with fluorophore-conjugated antibodies. Cells were stained with antibodies against the following molecules (clone, conjugate; source): CD45 (30-F11, APC-Cy7, BD), CD3 (145-2C11, BV786; BD), CD3 (145-2C11, BUV395; BD), CD4 (RM4-5, BV605; BD), CD4 (GK1.5, APC-Cy7; BD), CD8 (53-6.7, AF700; BioLegend),

TCRγδ (GL3, PE-Cy7; BioLegend), CD44 (IM7, APC; BD), CXCR5 (2G8, Biotin; BD), and PD-1 (RMP1-30, PerCP-ef710; eBioscience). Streptavidin-BV605 (BD) was also used. Stained cells were resuspended in viability dye DAPI to exclude dead cells. IL-4 and IL-13 were detected by the IL-4AC and IL-13DR reporters in 4C13R mice. Data were acquired on a BD LSRFortessa SORP flow cytometer (Becton Dickinson, San Jose, CA, USA). Flow data were analyzed using FlowJo software (Treestar).

## Statistical Analysis and Graphics

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Statistical comparisons used are specified in figure legends.

# RESULTS

## Priming With HDM Allergen Induces the Development of Th2 Subsets Producing Only IL-4AC in the Lymph Node and Distinct IL-4AC$^+$, IL-4AC$^+$IL-13DR$^+$, or IL-13DR$^+$ Expression Profiles in the Ear Tissue

To follow the activation, differentiation, and migration patterns of allergen-induced Th2 subsets, 4C13R mice were primed intradermally (i.d.) in the ear pinnae with HDM. The kinetic studies showed IL-4AC$^+$ CD4 T cells to be maximally elevated in the ear draining lymph node on days 5–9 after priming (Figures S1A,B in Supplementary Material), therefore ear lymph nodes and ear tissue were examined on day 7 for the appearance of IL-4AC- and IL-13DR-expressing CD4 T cells. CD4 T cells were defined as CD45$^+$CD3$^+$CD4$^+$CD8$^-$TCRγδ$^-$ cells (Figures S2A,B in Supplementary Material), thus excluding any TCRγδ$^+$ intraepithelial lymphocytes found in the ear tissue. HDM priming resulted in significant infiltration of CD4 T cells into these sites over the 7-day period, with a sixfold increase in the lymph node to $2.6 \times 10^6$ CD4 cells, while immunized ears had $2.9 \times 10^4$ CD4 cells compared with very few in naive mice (**Figures 1A,C**). The lymph node and ear tissue CD4$^+$ T cells displayed distinctly different Th2 cytokine profiles, which we could observe using the *Il4* and *Il13* gene locus directed expression of the AmCyan and DsRed fluorescent reporters, respectively (**Figures 1B,D–F**). In the ear draining lymph node, only IL-4AC-expressing CD4 Th2 cells could be detected, whereas in the ear tissue three distinct CD4 T cell subpopulations were found; IL-4AC single positives, IL-4AC/IL-13DR double positives and IL-13DR single positives. Comparison of the reporter median fluorescent intensities (MFIs) for each of these ear tissue-derived CD4 T cell subsets reveals that the double-positive CD4 Th2 cells have a higher MFI for both IL-4AC and IL-13DR reporters than do the single positives (**Figure 1G**), reflecting a higher degree of activation and the potential for producing a greater amount of cytokine on a per cell basis. As reported previously (6) *in vivo* generated IL-4 reporter positive cells expressed high levels of CD44, interestingly the IL-13DR single-positive CD4 T cells demonstrated the highest MFI level for CD44 expression (**Figure 1H**), indicating they

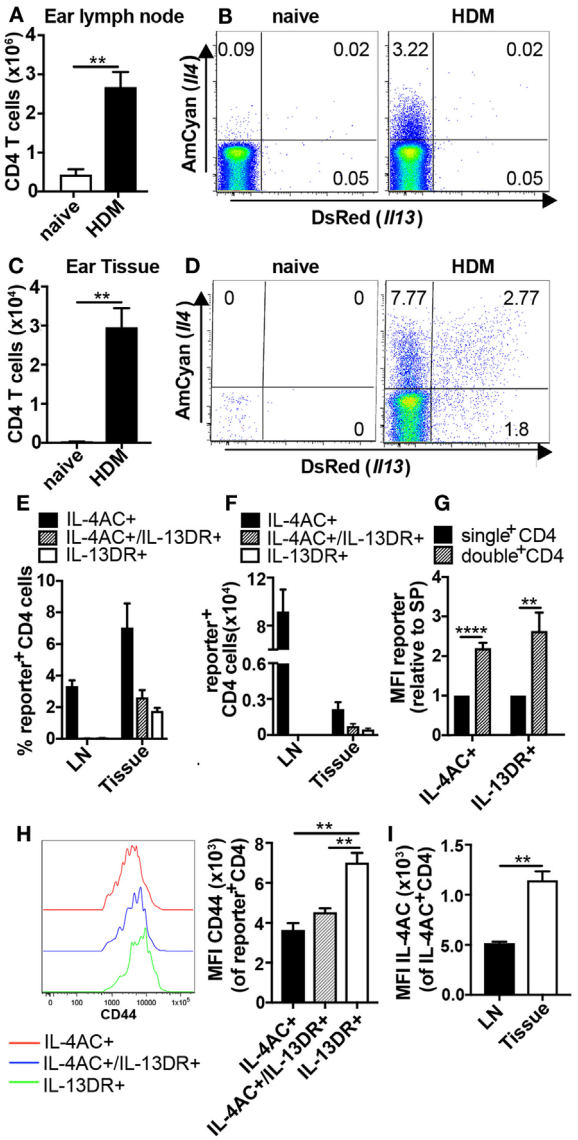


**FIGURE 1** | Priming with house dust mite (HDM) allergen induces the development of Th2 subsets producing only IL-4AC in the lymph node and distinct IL-4AC, IL-4AC/IL-13DR, or IL-13DR expression profiles in the ear tissue. 4C13R transgenic mice were challenged with 200 μg HDM i.d. in the ear. Ear draining lymph nodes and ear tissue were harvested 7 days later, and the presence of IL-4AC- and IL-13DR-expressing CD4 Th2 cells examined by flow cytometry. **(A,C)** Number of CD4 T cells in ear lymph node and ear tissue. **(B,D)** Concatenated FACS plots of CD4 T cells from naïve and HDM primed 4C13R transgenic mice showing IL-4AC$^+$ and IL-13DR$^+$ cells. **(E)** Proportions and **(F)** numbers of IL-4AC$^+$, IL-4AC$^+$/IL-13DR$^+$, and IL-13DR$^+$ subsets in the ear lymph node and ear tissue. **(G)** Median fluorescent intensity (MFI) of IL-4AC and IL-13DR reporters expressed in single reporter$^+$ vs double reporter$^+$ CD4 Th2 cells in the ear tissue (relative to MFI of single-positive cells). **(H)** MFI of CD44 expression on IL-4AC$^+$, IL-4AC$^+$/IL-13DR$^+$, and IL-13DR$^+$CD4$^+$ Th2 cells in the ear tissue. 96–98% of the reporter positive Th2 cells were CD44$^+$ **(I)** MFI of IL-4AC expression in IL-4AC$^+$ CD4 cells from ear lymph node and ear tissue. **(A–G,I)** Data from an experiment ($n$ = 3–5) representative of nine lymph node experiments and seven ear tissue experiments. **(H)** Results from a single experiment ($n$ = 3), representative of three experiments. Data show mean + SEM (**$p \leq 0.01$ and ****$p \leq 0.0001$ two-tailed $t$-test).

had a greater degree of activation than the IL-4AC- or IL-4AC/IL-13DR-expressing cells. Interestingly the tissue sourced IL-4AC$^+$ CD4 cells have a greater MFI of IL-4AC potentially reflecting their potential for higher levels of gene expression from the *Il4* locus than those CD4 T cells sourced from the lymph node (**Figure 1I**). Priming of mice with dead L3 *Nb* larvae in the ear stimulated a similar pattern of IL-4AC and IL-13DR expression by CD4 T cells in lymph node and tissues to that seen in HDM primed mice (Figures S3A–G in Supplementary Material).

To determine if the IL-4AC-expressing Th2 cells in the ear lymph node have the potential to produce IL-13DR upon *in vitro* restimulation, ear lymph node cells were harvested 7 days post HDM priming, and the whole lymph node cell population was cultured on anti-CD3 for 19 h. Stimulated CD4 cells expressed a similar proportion of IL-4AC as they did *ex vivo* (2.7%), while the percentage of CD4 T cells expressing IL-4AC cultured without anti-CD3 reduced over the time of culture (1.5%). Restimulation of the CD4 T cell subsets did not induce any further IL-13DR expression above background levels, indicating that the failure to detect IL-13DR expression in the lymph node was not due to lack of activation but rather the level of differentiation of the IL-4AC$^+$ CD4 T cells (Figures S4A–C in Supplementary Material).

These data demonstrate that following allergen priming in the ear tissue, distinct Th2 subsets develop in the draining lymph node and tissues. Specifically, CD4 T cells become committed to IL-4AC expression in both lymph node and tissues while the commitment of CD4 T cells to IL-13DR expression only occurs in the tissue.

## Both Tfh Cells and Th2 Cells Contribute to IL-4 Expression in the Lymph Node After HDM Challenge

As many studies have shown T follicular helper (Tfh) cells to be a dominant source of IL-4 in the lymph node (21–23), we sought to clarify the identity of the IL-4-expressing CD4 T cells observed in the ear lymph nodes of HDM primed mice. In analyzing the lymph node-derived IL-4AC$^+$ CD4 T cells, we identified both CXCR5$^+$PD-1$^+$CD4$^+$ (Tfh) and CXCR5$^{lo}$PD-1$^{lo}$, CXCR5$^+$PD-1$^{lo}$, and CXCR5$^{lo}$PD-1$^+$CD4$^+$ (Th2) populations (**Figure 2A**). Approximately 40% of the IL-4AC$^+$ cells were Tfh cells as defined by PD-1 and CXCR5 expression while the remaining were Th2 cells (**Figure 2B**). Gating on IL-4AC$^+$ Tfh and IL-4AC$^+$ Th2 cells as shown in **Figure 2C** allowed us to compare these two populations, showing the IL-4AC$^+$ Tfh cells to have a higher level of IL-4AC expression compared with the IL-4AC$^+$ Th2 cells (**Figure 2D**). In addition, the IL-4AC$^+$ Tfh cells showed a higher level of CD44 expression than the IL-4AC$^+$ Th2 cells (**Figure 2E**). The biological impact of this statistically significant difference is unclear.

Thus, it would appear from these data that following HDM introduction to the skin, CD4 T cells in the draining lymph node become committed to activated Tfh and Th2 phenotypes some of which express IL-4 and contribute to IL-4 production in the ear draining lymph node. Interestingly, Tfh cells also expressed higher levels of the IL-4AC reporter, perhaps suggesting a higher capacity to secrete IL-4.

## IL-4 Plays a Role in the Development of IL-4AC-Expressing Lymph Node Th2 Cells but Not IL-4AC-Expressing Tfh Cells

We next sought to investigate the role of IL-4 in the development of IL-4AC-expressing CD4 T cell populations in the draining lymph nodes of mice primed i.d. with HDM allergen. CD4 T cells from HDM primed IL-4$^{-/-}$ mice had a reduced proportion of IL-4AC-expressing CD4 cells compared with IL-4$^{+/+}$ mice (**Figure 3A**). Distinguishing Tfh and Th2 populations within the IL-4AC$^+$ cell subset reveals a shift in the proportion of these two subsets in the absence of IL-4, with a decrease of IL-4AC$^+$ Th2 cells and a corresponding increase in IL-4AC$^+$ Tfh cells (**Figure 3B**). Analysis of overall numbers shows that while the numbers of IL-4AC$^+$ Tfh cells are not compromised by the lack of IL-4, the development of IL-4AC$^+$ Th2 cells was inhibited by 50% in IL-4-deficient mice, illustrating partial dependence of this subset on IL-4 (**Figure 3C**). The MFI of IL-4AC expression was reduced in both IL-4AC$^+$ Tfh and Th2 subsets from the IL-4$^{-/-}$ mice (**Figure 3D**), suggesting that IL-4 positively regulates *Il4* expression in CD4$^+$ T cells. By contrast, the IL-4AC$^-$ Tfh and IL-4AC$^-$ non-Tfh populations survived better in IL-4-deficient conditions (Figures S5A,B in Supplementary Material).

Thus, it appears that the number of IL-4AC$^+$ Tfh cells in the lymph node is independent of IL-4, while Th2 CD4 cells in the ear lymph node are partially dependent on IL-4 for their development.

## IL-4 Is Required for the Development of IL-4AC- and IL-4AC/IL-13DR-Expressing Th2 Subsets, but Not IL-13DR-Expressing CD4$^+$ T Cell Subset, in Ear Tissue

We next studied the role of IL-4 in the development of Th2 subsets in the ear tissue following priming with either HDM or *Nb* allergens. As observed in **Figure 1**, three subsets of IL-4AC- or IL-13DR-expressing Th2 cells were identified in the ear after priming with HDM (**Figures 4A,B**) or dead *Nb* (**Figures 4F,G**). In both primary immunization models, the number of IL-13DR single-positive Th2 cells was not affected by the absence of IL-4 (**Figures 4C,H**), nor was there a reduction in the amount of IL-13DR they expressed as determined by MFI (**Figures 4E,J**). Although the proportion of IL-13DR Th2 cells was higher in IL-4$^{-/-}$ mice primed with non-viable Nb compared with IL-4-sufficient controls (**Figure 4G**), analysis of the total cell numbers revealed no difference (**Figure 4H**), due to the reduced overall numbers of CD4$^+$ T cells present in the IL-4$^{-/-}$ mouse (data not shown), suggesting that the higher proportion was simply due to the lack of other cell subsets in the IL-4$^{-/-}$ ear tissue. By contrast, the number of IL-4AC single-positive CD4 cells that appeared in the allergen primed ear tissue was reduced 8-fold in HDM immunized and an even greater 16-fold in *Nb* immunized 4C13R-IL-4$^{-/-}$ mice (**Figures 4C,H**). The IL-4AC/IL-13DR double reporter-expressing Th2 subset was also dependent on IL-4, with a threefold reduction in their levels observed in 4C13R-IL-4$^{-/-}$ mice in both models. Even though similar proportions of these cells were found in dead *Nb* immunized IL-4$^{+/+}$ and IL-4$^{-/-}$ mice (**Figure 4G**), their numbers were reduced in the IL-4-deficient

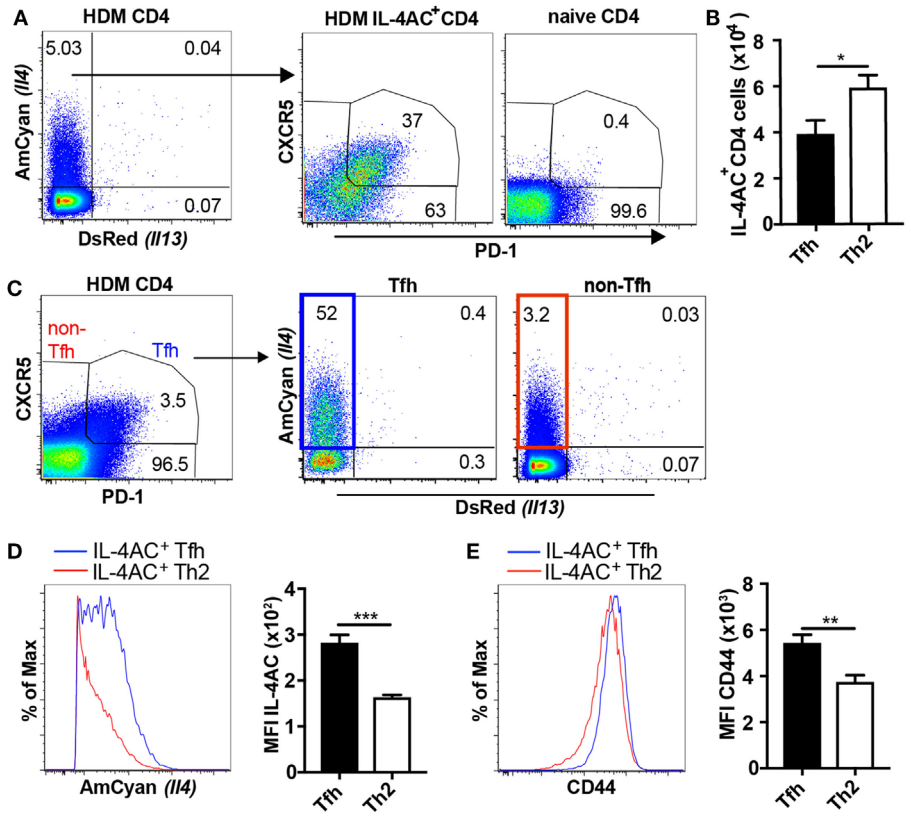


**FIGURE 2** | Both T follicular helper (Tfh) cells and Th2 cells contribute to IL-4AC expression in the lymph node after house dust mite (HDM) challenge. 4C13R transgenic mice were treated with 200 μg HDM i.d. in the ear. The ear draining lymph nodes were harvested 7 days posttreatment and analyzed by flow cytometry. **(A)** FACS plots showing the proportion of IL-4AC$^+$ CD4 T cells and then the proportion of CXCR5$^+$PD-1$^+$ Tfh and CXCR5$^{lo}$PD-1$^{lo}$, CXCR5$^+$PD-1$^{lo}$, and CXCR5$^{lo}$PD-1$^+$ Th2 cells within this population. **(B)** Numbers of IL-4AC$^+$ Tfh and IL-4AC$^+$ Th2 CD4 T cells. **(C)** FACS plots showing the proportion of CXCR5$^+$PD-1$^+$ Tfh and CXCR5$^{lo}$PD-1$^{lo}$, CXCR5$^+$PD-1$^{lo}$, and CXCR5$^{lo}$PD-1$^+$ non-Tfh CD4 T cells and then the proportion of IL-4AC$^+$ CD4 T cells within these populations. Median fluorescent intensity (MFI) of IL-4AC **(D)** and CD44 **(E)** expression on IL-4AC$^+$ Tfh and IL-4AC$^+$ Th2 CD4 T cells. **(A,C–E)** Data from a representative experiment ($n = 6$) of four experiments. **(B)** Data pooled from four experiments ($n = 23$). Data show mean + SEM (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ two-tailed $t$-test).

mice due to the reduced level of CD4 T cells in these mice as mentioned earlier. Furthermore, the MFI of IL-4AC in the IL-4AC$^+$ and IL-4AC$^+$/IL-13DR$^+$ Th2 cells was reduced by twofold in IL-4-deficient reporter mice (**Figures 4D,I**). Thus, as well as a significant reduction in the numbers of IL-4AC-expressing Th2 cells being detected in IL-4-deficient 4C13R-IL-4$^{-/-}$ mice, the gene expression from the *Il4* locus, as determined by examining reporter expression, was also reduced. As previously noted with IL-4-sufficient mice (**Figure 1G**), dual reporter IL-4AC$^+$/IL-13DR$^+$ Th2 cells from 4C13R-IL-4$^{-/-}$ mice also expressed greater levels of IL-4AC and IL-13DR reporters than the single reporter positive cells (**Figures 4D,E,I,J**).

To determine whether the distinct IL-4AC- and IL-13DR-expressing Th2 subsets could also develop at other tissue sites following antigen priming, we examined the Th2 subsets generated in the lung and BAL of mice responding to a live primary *Nb* infection, in which larvae infect the lung before migrating to the gut. We observed in both lung tissue and airways (BAL) the same three CD4$^+$ IL-4AC-, IL-4AC/IL-13DR-, and IL-13DR-expressing Th2 subsets (Figures S6A,C in Supplementary Material). The appearance of the IL-4AC CD4$^+$ Th2 subset was found to be significantly reduced in IL-4-deficient mice compared with IL-4-sufficient controls, the IL-4AC/IL-13DR and the IL-13DR CD4$^+$ Th2 subset were not significantly affected by IL-4 deficiency (Figures S6B,D in Supplementary Material).

In summary, The IL-4AC- and IL-13DR-expressing Th2 subsets that appear in ear tissues following antigen priming show differential requirements for IL-4, with IL-4AC$^+$ and IL-4AC$^+$/IL-13DR$^+$ Th2 cells being IL-4 dependent while the IL-13DR-expressing CD4 Th2 subset was not affected by the absence of IL-4.

## Reduction in IL-4 Availability Through *Il4* Gene Hemizygosity Has a Partial Effect on Development of IL-4AC- and IL-4AC/IL-13DR-Producing T Cell Subsets

The profound effect of complete IL-4 deficiency on the development of IL-4AC- and IL-4AC/IL-13DR-expressing Th2 subsets in the skin tissue and lung, led us to wonder what would be the effect

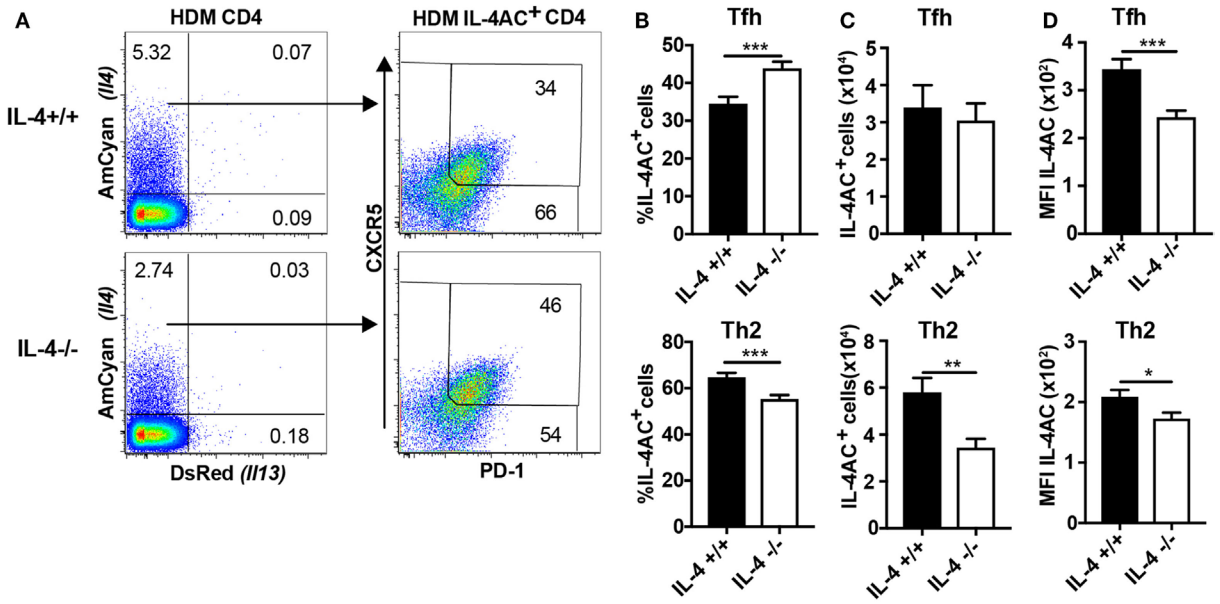


**FIGURE 3** | IL-4 plays a role in the development of IL-4AC-expressing lymph node Th2 cells but not IL-4AC-expressing T follicular helper (Tfh) cells. 4C13R-IL-4$^{+/+}$ and 4C13R-IL-4$^{-/-}$ mice were treated with 200 μg house dust mite (HDM) i.d. in the ear. The ear draining lymph nodes were harvested from mice 7 days posttreatment, and lymph nodes were analyzed by flow cytometry. **(A)** FACS plots showing the proportion of IL-4AC$^{+}$ CD4 T cells and then the proportion of CXCR5$^{+}$PD-1$^{+}$ Tfh and CXCR5$^{lo}$PD-1$^{lo}$, CXCR5$^{+}$PD-1$^{lo}$, and CXCR5$^{lo}$PD-1$^{+}$ Th2 cells within this population. **(B)** Proportion of IL-4AC$^{+}$CD4 T cells that are Tfh or Th2 cells. **(C)** Numbers of IL-4AC$^{+}$ Tfh and IL-4AC$^{+}$ Th2 CD4 T cells and **(D)** median fluorescent intensity (MFI) of IL-4AC of IL-4AC$^{+}$ Tfh and IL-4AC$^{+}$ Th2 CD4 T cells. **(A)** FACS plots from a representative experiment ($n = 6$). **(B,C)** Data pooled from three experiments ($n = 18$). **(D)** Data from a representative experiment ($n = 6$). Data show mean + SEM (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ two-tailed $t$-test).

of a partial *Il4* gene deletion such as that seen in IL-4 hemizygous mice. IL-4-sufficient (4C13R-IL-4$^{+/+}$), IL-4 hemizygous (4C13R-IL-4$^{+/-}$), and IL-4-deficient (4C13R-IL-4$^{-/-}$) mice were primed with HDM and their ear draining lymph nodes and ear tissue examined after 7 days. As reported in **Figure 3**, IL-4AC$^{+}$ Tfh cells in the lymph node were not dependent on IL-4, whereas levels of IL-4AC$^{+}$ Th2 cells were halved when IL-4 was not available (**Figure 5A**). Contrary to the IL-4-deficient system, the number of IL-4AC$^{+}$ Th2 cells in the hemizygous lymph nodes were the same as those in IL-4 wild-type mice indicating that even with reduced IL-4 availability the IL-4AC$^{+}$ Th2 cell response to HDM in the draining lymph node is capable of full development. In the tissue, however, the hemizygous *Il4* condition had a far more striking effect on the development of the IL-4AC$^{+}$ Th2 subset, with a five-fold reduction in cell numbers to levels only twofold greater than in the absence of IL-4 (**Figure 5B**). The reduction of the IL-4AC$^{+}$/IL-13DR$^{+}$ Th2 subset in the ear was less pronounced and, as was seen in **Figure 4**, the IL-13DR$^{+}$ Th2 cells were independent of IL-4 for their appearance in ear tissue. The disproportionate effect that the loss of half the wild-type IL-4 has on the IL-4AC$^{+}$ and the IL-4AC$^{+}$/IL-13DR$^{+}$ Th2 subsets in the ear reinforces the importance of IL-4 in the development of this distinct CD4 Th2 effector subset in the tissue microenvironment of the skin.

## DISCUSSION

We sought to clarify the role of IL-4 in the differentiation, migration, and accumulation of CD4$^{+}$ T cells following a primary response to allergen. We followed the number of IL-4- and IL-13-expressing CD4 T cells that appear in the draining lymph node and subsequent type 2 inflammatory response in the skin or lung following intradermal priming with allergen or parasite infection, respectively. We confirm that during the 7-day priming period the appearance of IL-4-expressing Tfh cells in the lymph node is not dependent on IL-4, while the appearance of IL-4-expressing Th2 cells in the lymph node is partially affected by the absence of IL-4. We describe for the first time the profound requirement for IL-4 in regulating the appearance of IL-4- and IL-4/IL-13-expressing Th2 subsets in the skin tissue 7 days following allergen priming. A similar IL-4 response profile was found for IL-4-producing Th2 cells in the lungs of *Nb* challenged mice. Surprisingly, we identified a unique CD4 T cell subset in the skin that was induced by both allergen priming in the skin and parasite infection in the lung that was committed solely to IL-13 expression and was completely independent of IL-4. Although normally IL-13 expression in CD4 T cells is linked to IL-4 expression and it is viewed as one of the canonical Th2 cytokines which is regulated by IL-4, a previous study using IL-4/IL-13 reporter mice identified that IL-13 expression occurs in lung tissue but not lymph nodes and that IL-4 and IL-13 expression is not always linked (23). Here, using our HDM skin priming model, we complement this data with confirmation in the ear skin tissue and its draining lymph node of the confinement of IL-13-expressing Th2 cells to the tissue compartment, while IL-4-expressing Th2 subsets reside in both lymphoid and non-lymphoid tissues. Furthermore, our finding that *in vitro* restimulation did not elicit IL-13 expression

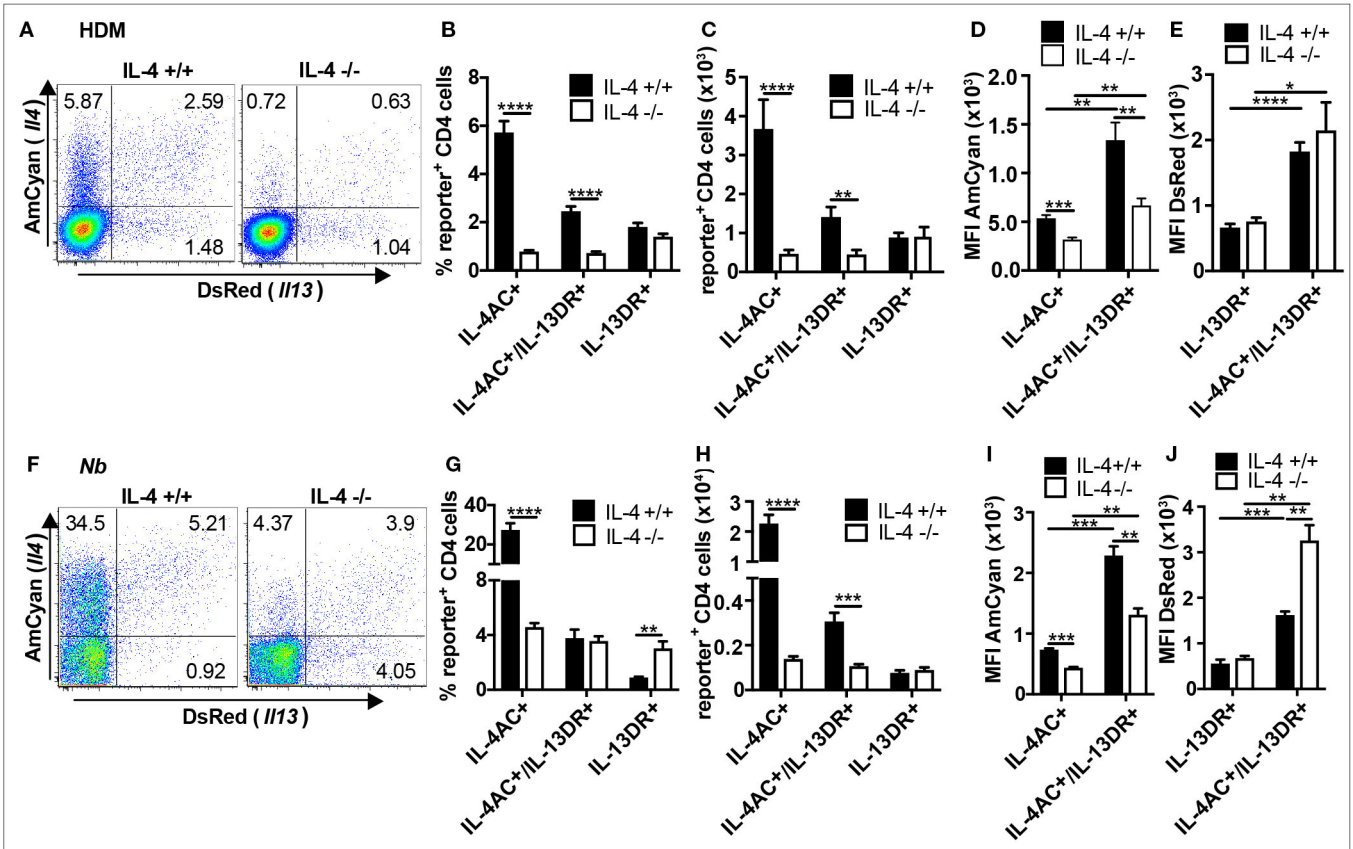


**FIGURE 4** | IL-4 is required for the development of IL-4AC- and IL-4AC/IL-13DR-expressing Th2 subsets but not the IL-13DR-expressing CD4+ T cell subset in ear tissue. 4C13R-IL-4$^{+/+}$ and 4C13R-IL-4$^{-/-}$ mice were treated with either **(A–E)** 200 μg house dust mite (HDM) or **(F–J)** 600 dead L3 *Nippostrongylus braziliensis* (*Nb*) i.d. in the ear pinnae. Ear tissue was harvested 7 days posttreatment. Tissues were analyzed to determine **(A,B,F,G)** proportion of IL-4AC+ and IL-13DR+ CD4 T cells, **(C,H)** number of IL-4AC+ and IL-13DR+ CD4 T cells, and **(D,E,I,J)** median fluorescent intensity (MFI) of IL-4AC and IL-13DR for each of the Th2 cell subsets. Data representative of seven experiments ($n$ = 35–37) for HDM and two experiments ($n$ = 6) for dead *Nb*. **(A)** FACS plots concatenated from a single representative experiment ($n$ = 6). **(B,C)** Data pooled from seven experiments ($n$ = 37). **(D,E)** Data from a single representative experiment ($n$ = 6). **(F)** FACS plots concatenated from a single representative experiment ($n$ = 3). **(G,H)** Data pooled from two experiments ($n$ = 6). **(I,J)** Data from a single representative experiment ($n$ = 3). Data show mean + SEM (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ two-tailed $t$-test).

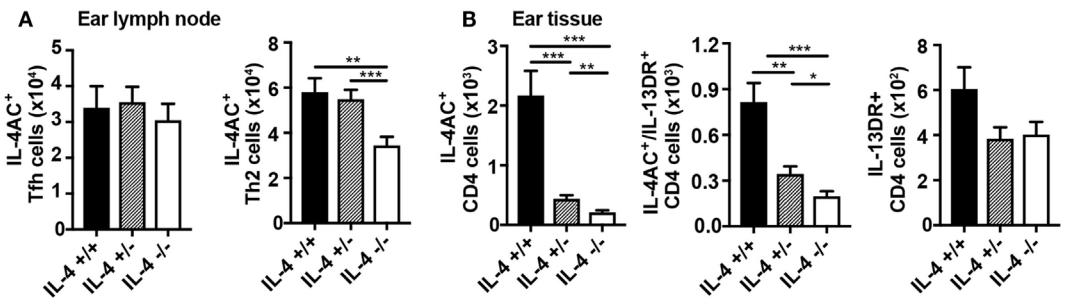


**FIGURE 5** | Reduction in IL-4 availability by *Il4* gene hemizygosity has a partial effect on the development of IL-4AC- and IL-4AC/IL-13DR-producing T cell subsets. 4C13R-IL-4$^{+/+}$, 4C13R-IL-4$^{+/-}$, and 4C13R-IL-4$^{-/-}$ mice were treated with 200 μg house dust mite i.d. in the ear pinnae. Ear draining lymph nodes and ear tissue were harvested from the mice 7 days posttreatment. **(A)** Lymph nodes were analyzed to determine the number of IL-4AC+ T follicular helper (Tfh) and IL-4AC+ Th2 CD4 T cells. **(B)** Ear tissue was analyzed to determine the number of reporter+ Th2 CD4+ T cells. **(A)** Data pooled from three experiments ($n$ = 18) for ear lymph node and **(B)** three experiments ($n$ = 15) for ear tissue. Data show mean + SEM (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ two-tailed $t$-test).

in CD4 T cells derived from the draining lymph node of HDM ear immunized mice confirms that the failure to detect IL-13 is not a sensitivity issue of the reporter system but rather whether the CD4 T cells have received the appropriate signals for activation of the *Il13* gene. The identification in the tissues of primed mice of a unique subset of CD4 T cells that only expressed IL-13 and

whose appearance was independent of IL-4 was surprising. When viewed in the context of recent work by others (8, 24) and our recent data showing that TSLP can regulate the development of an IL-13-producing CD4 Th2 subset (25), it becomes clear that other factors such as tissue alarmins are likely able to regulate or act as a tissue checkpoint for a IL-13-producing Th2 subset in the tissues.

T follicular helper cells have been shown to be a significant source of IL-4 in the lymph nodes during the Th2 response (21). In our studies following HDM priming of the ear skin, we find that both Tfh and Th2 cells contribute to IL-4 expression in the ear lymph node, although the Tfh cells had a greater degree of activation and made a greater amount of IL-4 than their Th2 counterparts, perhaps to aid their role helping B cells to class switch to IgE and IgG1 production. Tfh cells are traditionally thought to reside in the B cell follicle of the lymph nodes, but recent research has suggested that additionally Tfh cells may be precursors of Th2 cells subsequently found in the tissues (26, 27). Using a multiple prime and challenge lung model, Ballesteros et al. showed that HDM sensitization induced IL-4-committed Tfh cells in the lymph node which developed into IL-4- and IL-13-producing effector Th2 cells in the lung upon HDM challenge (26). Other research has identified a subset of Th2 promoting IL-21-expressing Tfh cells, distinct from Th2 cells, found in both the lymph node and lung tissue (27). Whether or not the Tfh cells seen in the ear lymph node in our ear HDM priming model are distinct from the ear tissue Th2 subsets remains to be determined. In addition, the link between the IL-4$^+$ Th2 subset seen in the lymph node and those Th2 subsets in the ear is unclear and subject to further research. It is probable that IL-4-expressing Th2 cells migrate from the lymph node to the ear tissue where they produce additional Th2 cytokines such as IL-5 and IL-13 for Th2 effector functions, perhaps under response to local damage elicited tissue signals (8). Of the IL-4-producing CD4$^+$ subsets in the lymph node, the Th2 subset is more likely than the Tfh cells to be the precursors of the tissue Th2 subsets, as both the Th2 subsets, but not Tfh cells demonstrate a dependence on IL-4 for their development. It should be noted though that the MFIs of the IL-4AC reporter were significantly reduced in both the Tfh and Th2 lymph node CD4 T cells subsets indicating that the levels of IL-4 may be regulated through indirect means such as signaling by B cells or dendritic cells.

That the development of IL-4-expressing Tfh cells is independent of IL-4 adds to current recognition of Tfh cells being a unique subset distinct from traditional Th2 cells. Development of Tfh cells instead depends on IL-6, IL-21, STAT3, and BCL6 and not on other cytokine or transcription factors necessary for formation of other T helper subsets (28–30), and it has been reported that the Tfh cell transcription factor BCL6 can limit the activities of Th2 cells (31). However, it should be noted that studies by others show that the IL-4-producing Th2 subset in the lymph node is not affected by BCL6 deletion indicating that the Tfh subset may not be a precursor for lymph node Th2 (32). Our studies identify a second IL-4-expressing CD4 T cell subset in the lymph node that does not express Tfh markers but whose expression of IL-4 appears to be partially IL-4 dependent. In support of our finding, recent studies have shown that IL-4Rα knockout mice exhibited a significantly compromised IL-4-expressing Th2 response in the gut draining mesenteric lymph node of *H. polygyrus*-infected mice (33) As IL-4Rα is a component of the receptors *via* which both IL-4 and IL-13 act, this could be attributed to the inability of IL-4 (or IL-13) to act *via* its receptor. In our studies, generation of IL-4AC-expressing lymph node Th2 cells was not effected in mice lacking one IL-4 allele and was only reduced by half in the complete absence of IL-4, thus showing only a partial requirement for IL-4. This suggests that although IL-4 contributes to Th2 differentiation in the lymph node other factors are likely involved, such as the quality of the TCR signal and co-stimulation, Th2 promoting miRNAs, signaling pathways that promote GATA3 expression, and other Th2 promoting cytokines like TSLP, IL-33, and IL-25 (34–39). In contrast to our present findings, previous research using G4 reporter mice has indicated that IL-4 was not required to generate IL-4-expressing Th2 cells in the lymph nodes of *Nb*-infected mice (6). However, in these studies only G4/IL4 heterozygous mice in which IL-4 was produced from only one allele could be compared with IL-4-deficient G4/G4 mice in the various allergen and parasitic models and no difference in numbers of GFP-expressing Th2 cells was seen. The use of our dual IL-4, IL-13 4C13R reporter mice in this study, where the reporters are inserted into a bacterial artificial chromosome leaving the endogenous cytokines intact, has enabled a fuller comparison of the effect of the IL-4-sufficient, IL-4 heterozygous, and the IL-4-deficient states on IL-4-expressing CD4 T cell development. In agreement with the previous work, no significant difference was seen between numbers of Th2-differentiated cells in IL-4$^{+/-}$ and IL-4$^{-/-}$ mice in the lymph node, but the additional comparison with the IL-4$^{+/+}$ mice enabled the conclusion that differentiation of Th2 cells is in fact partially dependent on IL-4. Other research comparing NP-OVA/Alum ear immunized IL4$^{+/+}$, IL4$^{+/-}$, and IL-4$^{-/-}$ 4C13R mice also showed IL-4-expressing Th2 cells in the lymph node to not be dependent on IL-4 (20). In another study investigating IL-4- and IL-13-expressing cells arising in response to a model of OVA-induced lung allergy (40), dual reporter *Il4*$^{+/eGFP}$ *Il13*$^{+/Tom}$ mice were used, which due to the insertion of the reporter constructs, are hemizygous for the IL-4 and IL-13 cytokines. They found that IL-4eGFP-expressing CD4$^+$ T cells were largely absent from the lung while in the mediastinal lymph node they were present and major contributors to IL-4 production. This replicates what we have seen in IL-4 hemizygous mice in our current study (**Figure 5**), where IL-4AC$^+$ Th2 cells are not sensitive to the partial loss of IL-4 in the lymph node but in the ear tissue are sensitive to even the hemizygous IL-4 state, with severely decreased numbers. The reliance on IL-4-knockin reporter mice fails to achieve an accurate representation of cytokine expression in a fully IL-4-sufficient environment, while the 4C13R reporter mice overcome this limitation. However, it should be noted that studies using gene expression reporter constructs do not necessarily give any indication of the protein levels that are produced by these Th2 cells and further studies would be required to determine whether the gene expression findings reported here translate into protein expression.

In addition to the IL-4-expressing Tfh subset, we have identified using IL-4AC and IL-13DR expression three subsets of CD4$^+$ T cells that would fall under the definition of a Th2 effector cell that is primed by allergen (or parasite antigens) in the draining lymph node and then migrate and expresses functional cytokines at the original antigen challenged tissue site. The IL-4- and IL-4/

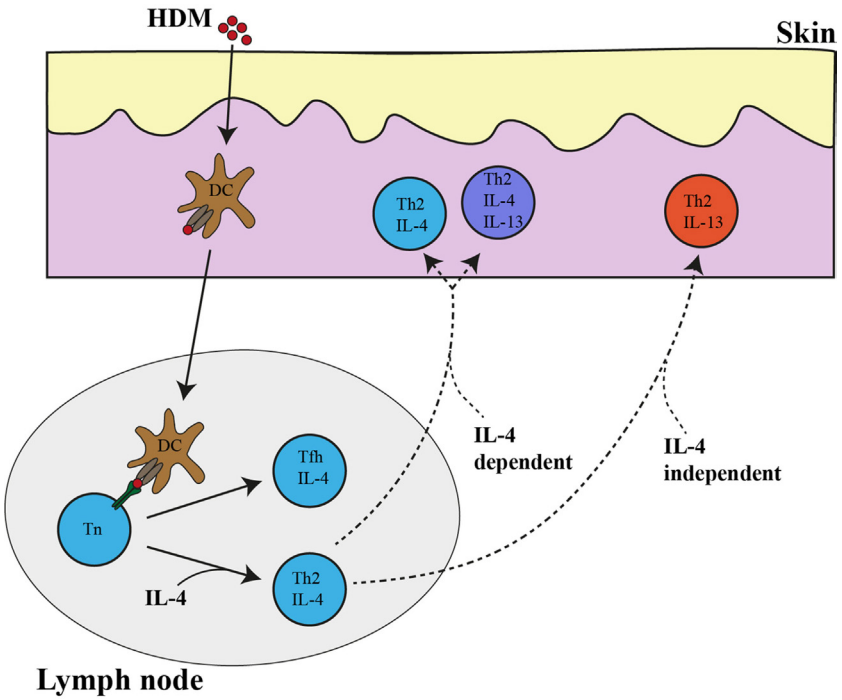


**FIGURE 6** | IL-4- and IL-13-expressing Th2 subsets in the lymph node and ear tissue after allergen priming. Following house dust mite (HDM) priming in the ear skin, dendritic cells take up allergen and transport it to the ear draining lymph node where they present it to naïve CD4 T cells. Naïve CD4 T cells differentiate into either (i) IL-4-expressing Th2 cells in a process that is partially dependent on IL-4 or (ii) IL-4 independent IL-4-expressing T follicular helper (Tfh) cells. IL-4 single-positive CD4 T cell and IL-4/IL-13 double-positive-expressing CD4 T cell subsets in the skin tissue are very dependent on IL-4 while IL-13 single-positive-expressing Th2 cells are independent of IL-4. The broken line represent the as of yet unproven but potential link between the lymph node Th2 cells and those in the tissue.

IL-13-expressing Th2 subsets are very dependent on IL-4 for their appearance in the tissues. Although outside the scope of this study, the requirement for IL-4 may be either to (i) allow differentiation/survival of differentiating Th2 subsets to *Il4* and *Il13* gene expression, (ii) regulate migration and extravasation of CD4 Th2 subsets to antigen challenged tissue sites or (iii) its continued presence is required when reactivated with antigen at the tissue site for full differentiation to a IL-4- or IL-4/IL-13-expressing Th2 effector to occur. Also, our observation that a distinct IL-13-expressing CD4 T cell subset appears in tissues following antigen challenge that is not regulated by IL-4 may reflect the ability of specific tissue-derived alarmins to program specific cytokine expression patterns. Taken together, a picture emerges whereby the differentiating Th2 cell appears to be regulated by multiple checkpoints at distinct tissue sites (**Figure 6**). The lymph node appears to be a place for antigen priming and activation of naïve T cells to Th2 precursors, some of which sequester to the B cell areas of the lymph node to drive B cell differentiation and the others to quickly circulate throughout the body and home to the tissue sites challenged with priming antigens. IL-4 and specific tissue-derived alarmins and factors appear able at this point to direct the further differentiation of the Th2 precursors to distinct subsets of IL-4-, IL-4/IL-13-, and IL-13-expressing subsets. The value or relevance of each Th2 effector subset to the process of antigen/pathogen neutralization, clearance, and repair needs to be elucidated by further studies.

The development of reagents which can selectively interfere with the actions of Th2 cytokines is a potential therapeutic approach in the treatment of allergic disorders, and antibodies targeting the individual cytokines and their receptors have met with variable results [reviewed in Ref (41)]. Dupilumab that blocks IL4Rα, a subunit of both the IL-4 and IL-13 receptors, interferes with the action of both these cytokines and to date has had success in treating asthma and atopic dermatitis (42, 43). The ability of this drug to simultaneously target and block the functions of the lymph node and tissue Th2 cells, thus inhibiting humoral and cellular aspects of type 2-driven pathology is likely key to its success. Further insights into the nature of IL-4- and IL-13-producing Th2 subsets generated in response to allergic stimuli will beneficially further the understanding of the anti-allergic effects of these agents and contribute to their ongoing development.

## ETHICS STATEMENT

All animal procedures were approved by the Victoria University of Wellington Animal Ethics committee and performed in accordance with institutional guidelines.

## AUTHOR CONTRIBUTIONS

MP carried out the experiments, analyzed the data, and wrote the manuscript. GG, FR, and RK provided conceptual insights

and editing of the manuscript and GLG supervised the project. All authors provided feedback on the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01211/full#supplementary-material.

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REVIEW
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# IL-4 and IL-13 Receptor Signaling From 4PS to Insulin Receptor Substrate 2: There and Back Again, a Historical View

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In this historical perspective, written in honor of Dr. William E. Paul, we describe the initial discovery of one of the dominant substrates for tyrosine phosphorylation stimulated by IL-4. We further describe how this "IL-4-induced phosphorylated substrate" (4PS) was characterized as a member of the insulin receptor substrate (IRS) family of large adaptor proteins that link IL-4 and insulin receptors to activation of the phosphatidylinositol 3′ kinase pathway as well as other downstream signaling pathways. The relative contribution of the 4PS/IRS pathway to the early models of IL-4-induced proliferation and suppression of apoptosis are compared to our more recent understanding of the complex interplay between positive and negative regulatory pathways emanating from members of the IRS family that impact allergic responses.

**Keywords: interleukin-4, interleukin-13, interleukin-4 receptor α, interleukin-13 receptor alpha1 subunit, insulin receptor substrate, IL-4-induced phosphorylated substrate, allergy, macrophage activation**

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## FORWARD BY ACHSAH D. KEEGAN

Working with Dr. William E. Paul, known to all as "Bill," was an honor and a privilege. It was also a lot of fun. In the early 1990s his laboratory was energized by studies of Th2 differentiation, the composition of the receptor for IL-4 (and later IL-13), and mechanisms of signal transduction. These studies included the identification and initial characterization of a major target for tyrosine phosphorylation in cells treated with IL-4, the focus of this perspective. As fellows, working with (not for) Bill was like being a kid in the proverbial candy shop; we were only limited by our imagination and ability to work hard. Bill's enthusiasm for each project was infectious; he challenged all of us to think creatively and ask important questions. His scientific legacy is profound and timeless. Fascination with IL-4 signaling, starting with work in Bill's lab, continues today; co-authors Dr. Zamorano and Dr. Heller trained as postdoctoral fellows in my laboratory before starting their own programs, and Dr. Keselman is currently a fellow in Dr. Heller's lab. The latest research is leading to new and increasingly complex paradigms on pathway regulation with implications for the treatment of allergic diseases. And so on it goes.

## AN INTRODUCTION TO 4PS AND THE INSULIN RECEPTOR SUBSTRATE (IRS)

With the development of monoclonal antibodies capable of recognizing proteins phosphorylated on tyrosine (Y) residues, scientists were able to efficiently and consistently analyze patterns of tyrosine

phosphorylation induced by a variety of growth factors and cytokines (**Figure 1**) (1–4). Early studies performed in collaboration with Dr. Jacalyn Pierce and Dr. Ling-Mei Wang showed that IL-4 treatment of the mouse myeloid factor-dependent cell line (FDC)-P2 stimulated the highly robust tyrosine phosphorylation of a large molecular weight protein (~180,000 Da), visible in anti-phosphotyrosine western blots of anti-phosphotyrosine precipitates, while stimulation with IL-3 failed to do so (5). We initially termed this protein IL-4-induced phosphorylated substrate or "4PS." This phospho-protein was shown to associate with the p85 regulatory subunit of phosphatidyl-inositol (PI) 3′ kinase and with PI 3′ kinase enzyme activity.

Groups interested in the signaling pathways activated by insulin, including Dr. Kahn and Dr. White, had reported that insulin treatment of responsive cells led to the robust tyrosine phosphorylation of a large molecular weight protein (~185,000 Da) they termed insulin receptor substrate or "IRS" (6, 7). Intrigued by the similarity to 4PS, we directly compared the effects of IL-4 and insulin on tyrosine phosphorylation in FDC-P2 cells (8). Both induced the tyrosine phosphorylation of a protein with similar mobility on SDS-PAGE gels that was capable of interacting with the p85 regulatory subunit of PI 3′ kinase. Subsequent analysis of the phosphoproteins by V8 protease digestion revealed that the IL-4-induced tyrosine-phosphorylated substrate was similar to that phosphorylated in response to insulin and IGF-I suggesting that 4PS was related to IRS (8).

Dr. White's group cloned the cDNA for IRS from rat liver, and it was termed IRS1 (9). The IL-3-dependent murine cell line, 32D, expressing IRS1 as a result of transfection, was generated in Dr. Pierce's lab and used to show unequivocally that both IL-4 and insulin stimulated the tyrosine phosphorylation of IRS1 (10). This pathway was essential for the ability of IL-4 to stimulate 32D cell proliferation, and thus the concept that the 4PS/IRS pathway is required for proliferative responses was initiated. In later studies, it was observed that IL-13 also induced the tyrosine phosphorylation of 4PS (11) (**Figure 2A**); the potency of 4PS phosphorylation correlated with the proliferative response in human TF-1 cells (**Figure 2B**).

With the molecular characterization of IRS1 and development of IRS1-specific antibodies, it became clear that the 4PS protein observed in FDC-P2 cells was not IRS1 (8, 9). Polyclonal anti-IRS1 anti-serum weakly recognized 4PS in FDC lines, while two highly specific anti-IRS1 peptide antibodies were unable to precipitate 4PS. Thus, protein sequence for 4PS was obtained from anti-p85 precipitates of insulin-treated FDC-P2 cells (12). The sequence was used to generate probes to screen a cDNA library generated from FDC-P2 cells and obtain sequence for 4PS in 1995. 4PS was renamed IRS2 due to its similarity to IRS1 (12).

It is now known that IRS1 and IRS2 are members of a family of large adaptor proteins that participate in insulin, IGF-1, and IL-4 and IL-13 signaling (13). A variety of other growth factors and cytokines have also been shown to stimulate the phosphorylation

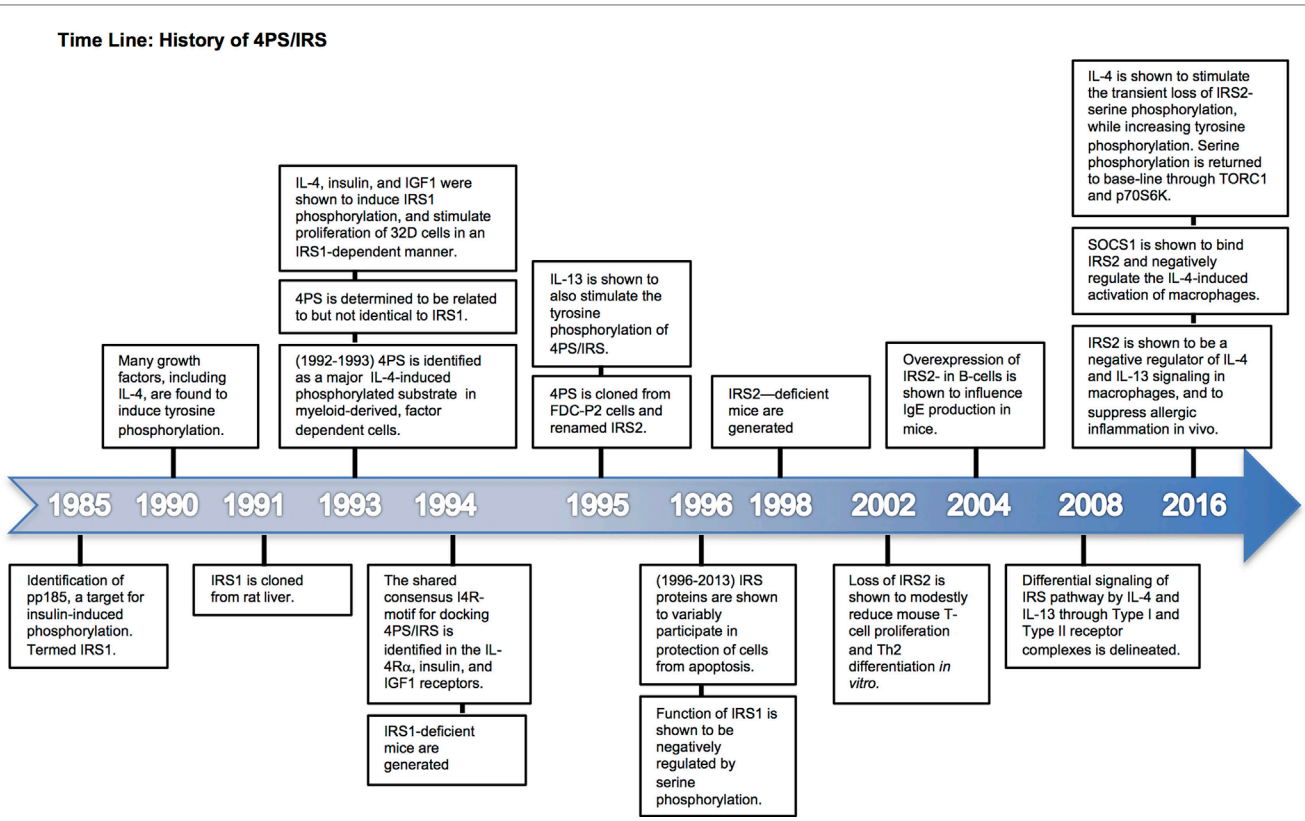


**FIGURE 1** | Time line of 4PS discovery and characterization. Major milestone discoveries are ordered and summarized based on the year(s) of their publication.

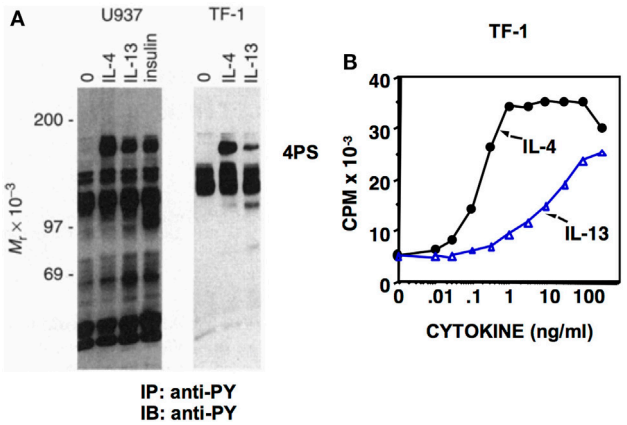


**FIGURE 2** | Induction of 4PS and cell proliferation by IL-4 and IL-13. **(A)** Human U937 and TF-1 cells were deprived of serum and growth factors for 2 h before treatment with human IL-4 (10 ng/ml), human IL-13 (250 ng/ml), or insulin (40 μg/ml) as indicated. Cell lysates were prepared and immunoprecipitated with the anti-phosphotyrosine antibody 4G10 followed by immunoblotting with 4G10. **(B)** TF-1 cells were incubated with the indicated doses of human IL-4 and human IL-13 for a total of 48 h. Cells were treated for the last 4 h of culture with [$^3$H]thymidine. Reprinted under copyright (1995) National Academy of Sciences, U.S.A. Keegan et al. (11).

of these signaling substrates (14). Both IRS1 and IRS2 can be tyrosine phosphorylated in response to IL-4 while other family members (including IRS3 or IRS4) do not appear to participate. Whether IRS1 or IRS2 or both are tyrosine phosphorylated after IL-4 stimulation depends on the cellular expression of each protein (15). Studies in 32D cells, which express neither IRS protein, revealed a positive contribution of either IRS1 or IRS2 to the IL-4-induced proliferative response (10). It was initially thought that IRS1 was predominantly expressed in non-hematopoietic cells, while IRS2 was highly expressed in cells of hematopoietic origin. However, there are exceptions to this paradigm, especially in epithelial cancers such as breast cancer (16). Furthermore, myeloid cells can express IRS1 with important functional activity as we discuss below (17). It is now appreciated that many cell types can express both family members, with differences in relative abundance that may be regulated (18).

Both IRS1 and IRS2 contain conserved amino terminal plexin homology domains and protein tyrosine binding (PTB) domains that bring these adaptors to the inner leaflet of the plasma membrane (19, 20) and interact with tyrosine-based target motifs (21), respectively. Both adaptors contain multiple tyrosines that have the potential to become phosphorylated, explaining their dominant representation in anti-phosphotyrosine immunoprecipitates. Three groups demonstrated that the Janus kinase (JAK) interacting with the cytoplasmic tail of the IL-4Rα chain, JAK1, is required for IL-4-induced tyrosine phosphorylation of IRS proteins (22–24). In collaboration with John O'Shea, we showed that IL-4 treatment lead to the activation of JAK3, while IL-13 treatment did not (11). IL-13 was shown to activate Tyk2 or in some cases JAK2 (25, 26). Both stimulated tyrosine phosphorylation of IRS2 (11).

Once phosphorylated, the tyrosine residues provide docking sites for SH2-domain-containing signaling molecules, such as the p85 subunit of PI 3′ kinase and the small protein adapter Grb2 (27). There are three tyrosines that act as p85 binding sites in IRS1 and two in IRS2 in the classic YXXM motif (13, 28). Binding of p85 to IRS proteins leads to activation of PI 3′ kinase activity and the subsequent activation of downstream signaling cascades such as the Akt pathway. The functional importance of the recruitment of the Grb2 adaptor is still unknown (27). Many other adaptor proteins have also been shown to associate with IRS1 or IRS2 including SHP-2 (also known as Syp, SH-PTP2) (29), PLC-γ (30), and SOCS proteins (31, 32), negative regulators of IL-4 signaling.

In addition to sites for tyrosine phosphorylation, both IRS1 and IRS2 have numerous potential sites for serine and threonine phosphorylation; several of these sites are unique to IRS1 and act as important modulators of functions as will be discussed in more detail in a later section (33). While well known as cytoplasmic adaptor proteins, IRS1 and IRS2 are not confined to the cytoplasm. Both can also translocate to the nucleus under certain conditions (viral/cellular transformation) and contribute to transcriptional activation or inhibition of particular genes (34–37).

## RECRUITMENT TO THE IL-4 RECEPTOR COMPLEX: WELCOME TO THE INSULIN/IL-4 RECEPTOR (I4R) MOTIF

In order to understand the mechanism by which IL-4 stimulated the tyrosine phosphorylation of 4PS/IRS and cellular proliferation, a series of deletion, mutagenesis, and pull-down studies were performed in Bill's lab in collaboration with Dr. Keats Nelms (38). The amino acids in the cytoplasmic tail of the IL-4Rα chain responsible for 4PS/IRS binding to the human IL-4 receptor were identified between amino acids 437 and 557. Furthermore, this sequence interval was necessary for IL-4 to stimulate proliferation of 32D-IRS1 cells. Within this interval, we identified a sequence motif homologous to sites within the insulin and IGF-I receptors previously shown to bind IRS1. We named this consensus motif [$^{488}$PL-$(X)_4$-NPXYXSXSD$^{502}$] the insulin and IL-4 Receptor (I4R) motif. The central tyrosine is critical for association of IRS proteins with the I4R motif of the IL-4Rα and for proliferation of transfected 32D-IRS1 cells (38, 39). The PTB domains of IRS1/2 recognize the core NPXY sequence when phosphorylated with influence of the amino acid residues in the −9, −8, and −7 (relative to the Y residue) positions (21, 39).

The importance of the I4R motif in dictating IL-4 receptor signaling was confirmed using domain transplant approaches (40). We generated chimeric receptors using a truncated IL-2 receptor β chain fused to the IL-4Rα domain containing the I4R motif (aa437–557) in wild type form or with the central Y residue mutated to F. Only chimeric receptors containing a wild-type I4R motif were able to mediate the tyrosine phosphorylation of IRS1 in response to IL-2.

As the IRS pathway was being characterized, contemporaneous work from several groups were on the trail of another protein tyrosine phosphorylated in response to IL-4 (41–44). This protein was identified as a member of the new (at the time) family of signal transducers and activators of transcription (STAT), and termed STAT6. The tyrosine phosphorylation of STAT6 induced

by IL-4 leads to its ability to bind to STAT-palindrome sequences found in the promoters of IL-4 responsive genes such as CD23 and regulate gene transcription. While working in Bill's lab, John Ryan showed that STAT6 was recruited to the IL-4Rα by any one of the three distinct amino acid motifs with the consensus sequence of GYKxF (45). Indeed, mutating the Y in these IL-4Rα sequences to F substantially diminished STAT6 phosphorylation in response to IL-4 and suppressed the majority of IL-4-induced responses. These STAT6 docking motifs were independent of the I4R motif. Thus, at the initial steps of signaling transduction, activation of the IRS and STAT6 pathways are independent of each other. Taken together, the published studies of the 1990s led to the conclusion that there were two major signal transduction pathways activated by IL-4. Models of the day showed that the STAT6 pathway regulated gene expression while the IRS pathway regulated cell proliferation (46–51). Later studies called this dichotomy into question as most IL-4-induced functions are greatly diminished or abrogated in STAT6-deficient mice (52–57).

## CONTRIBUTION OF IRS PROTEINS TO CELL SURVIVAL

The ability of IL-4 to regulate the survival of cells is one of the important and most investigated activity of this cytokine. Soon after its characterization, IL-4 was found to exert potent anti-apoptotic activity, preventing the apoptosis of multiple cell types under different pro-apoptotic signals (58). The molecular mechanisms that signal regulation of apoptosis by IL-4 have been widely studied. These studies established that IL-4 can signal various intracellular pathways able to regulate apoptosis. Among, the molecular machinery involved in this process, the IRS proteins were found to play an important active role in the regulation of apoptosis by IL-4 (59).

As noted above, early studies performed in cell lines lacking IRS proteins demonstrated that the IL-4-induced cell proliferation was dependent on these proteins (10). Similarly, later studies performed in these cells also demonstrated a principal role of IRS proteins in the protection of apoptosis by IL-4. Thus, we showed that expression of IRS1 in 32D cells enhanced the ability of IL-4 to protect them from apoptosis after IL-3 withdrawal (59). This observation was further supported by the fact that IL-4 was not able to prevent cell death in cells expressing the Y497F mutation within the I4R motif of the IL-4Rα. This mutation abrogated the ability of IL-4 to induce IRS proteins phosphorylation. The importance of the I4R motif in regulating apoptosis was also observed in chimeric receptors consisting of a truncated form of the IL-2 receptor, unable to signal protection from apoptosis, and different fragments of the IL-4Rα (60). Transplantation of the IL-4Rα domain containing the I4R motif to the truncated IL-2 receptor transferred the ability to activate IRS proteins and to signal protection from apoptosis. This was abrogated again by the mutation Y497F within the I4R motif. These studies demonstrated the importance of the I4R motif of the IL-4Rα and the IRS proteins in the regulation of apoptosis by IL-4. In spite of these observations, the regulation of apoptosis by IL-4 seems to be more complex. IL-4 can activate IRS-independent pathways, including STAT6, to prevent cell death since IL-4 could protect from apoptosis cells lacking IRS proteins, though less effectively that in cells expressing them (59–63).

The IL-13 receptor complex also contains the IL-4Rα (**Figure 3**), sharing, therefore, intracellular molecular pathways and biological functions with IL-4 including protection from apoptosis. However, the role of IRS proteins in IL-13 signaling protection from apoptosis has not been extensively investigated. Like IL-4, IL-13 is also able to signal IRS phosphorylation (11). However, the phosphorylation of IRS2 induced by IL-13 is much weaker than by IL-4 (64). This observation could help to explain the differential described effect of IL-4 and IL-13 in apoptosis. Thus, IL-13 could reduce apoptosis in peripheral B cells although it was less potent than IL-4 (65). Both cytokines appear to activate common pathways since their effect was not additive. It may be possible that they converge on IRS2 as it has been proposed that IL-13 prevents pancreatic beta cells from apoptosis through IRS2 signaling (66).

The ability of IRS proteins to signal protection from apoptosis is not restricted to IL-4. A number of studies have shown that insulin and IGF-1 promoted pancreatic beta cell development and survival through IRS2 signaling (67). It was observed that disruption of IRS2 produced diabetes in mice by affecting development and survival of beta cells (68). By contrast, overexpression of IRS2 could improve beta cell function by protecting them from apoptosis induced by D-glucose (69). Disruption of IRS2 has been demonstrated to impair peripheral insulin signaling promoting insulin resistance in liver and skeletal muscle (68).

The effects of the IRS adaptors in preventing cell death can be extended to other cell types including hepatic, muscular, or neuronal cells. IRS2 is the main effector of insulin in the liver. IRS2 signaling has been found necessary to mediate the survival effect of insulin in neonatal hepatocytes. In this case, insulin rescue of hepatocytes from apoptosis was aborted in cells lacking IRS2 (70). The introduction of IRS2 in these cells reconstituted the ability of insulin to prevent cell death. IRS2 is overexpressed in human and murine hepatocellular carcinoma, resulting in protection from apoptosis. In these cells, downregulation of IRS2 increased apoptosis (70, 71).

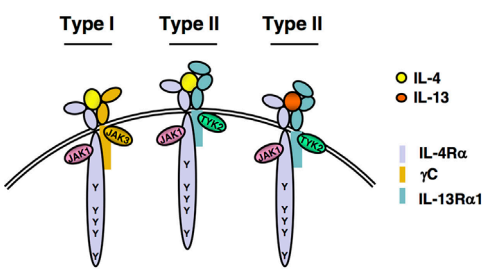


**FIGURE 3** | IL-4 and IL-13 receptors. A functional IL-4 receptor is composed of two transmembrane proteins. The IL-4Rα chain binds IL-4 with high affinity, leading to dimerization with the common gamma chain (γc) to form the Type I, IL-4 exclusive receptor complex or with the IL-13Rα1, to form the Type II IL-4 receptor complex. IL-13 binds to IL-13Rα1 with lower affinity, followed by heterodimerization with IL-4Rα to form the IL-13 Type II receptor complex. Following ligand binding and subunit heterodimerization, receptor-associated Janus Kinases (JAKs) become activated and phosphorylate any of the five highly conserved tyrosine residues found in the cytoplasmic tail of the IL-4Rα chain.

Given their ability to signal protection from apoptosis, it is not surprising that IRS proteins contribute to cancer development and progression. Numerous studies have implicated IRS proteins in the progression of several tumors including breast, colorectal, prostatic, hepatic, or gastric cancers (37, 72–80). It has been proposed that IRS proteins may play an important role in breast cancer by differentially regulating cell survival, proliferation, and motility (75, 81). Increased IRS1 abundance has been associated with breast cancer cell proliferation (16). Increased IRS1 expression has been reported in primary estrogen receptor α (ERα) + breast tumors and localized breast ductal carcinoma *in situ* (37, 82). Interestingly, IRS1 interacts with ERα, and in the nucleus regulates ERα transcription (34, 36, 83–85). Furthermore, estrogen regulates expression of IRS1, thus providing a positive regulatory pathway between estrogen and the IRS1 adaptor (86). In keeping with this relationship, low IRS1 expression was observed in poorly differentiated ERα-tumors (37). On the other hand, IRS2 expression is regulated by progesterone and is associated with metastasis (81, 87, 88). The expression of IRS2 was low in ductal carcinoma *in situ* but much increased in high grade invasive human breast tumors (37). Using mouse models of breast cancer, it was shown that overexpressing IRS2 lead to mammary hyperplasia, tumorigenesis, and metastasis (74). By contrast, IRS2-deficient mammary tumor cells were less invasive and more apoptotic than cells expressing IRS2 (89, 90). Interestingly, increased expression of IRS1, but not IRS2, may favor anticancer therapies. IRS1 expression sensitized MCF-7 cells to breast cancer chemotherapeutic agents, likely by affecting Annexin-2 cellular distribution (37). Similar findings were also observed in 32D myeloid cells (91). In these cells, overexpression of IRS1, but not IRS2, also enhanced their sensitivity to chemotherapy by enhancing Annexin-A2 expression. Surprisingly, coexpression of IRS2 suppressed sensitization of chemotherapy by IRS1, and altered the subcellular localization of IRS1 and Annexin-A2 from primarily cytoplasmic to primarily nuclear. These findings suggest that analysis of the relative expression of IRS proteins may be used to predict breast cancer progression and response to chemotherapy. In this regard, other authors have proposed that IRS-specific gene expression profiles could predict the response to anti-IGF therapy in breast cancer (76).

A recent meta-analysis indicates that the IRS2 rs1805097 polymorphism can be associated with the risk of developing colorectal cancer (77). The same polymorphism has been associated with susceptibility to gastric cancer (78). In prostate, it has been reported that the IRS2/IRS1 ratio was higher in malignant compared with benign prostate tissues (79). IRS2 was also found overexpressed in human and mouse hepatocellular carcinoma cells, and down regulation of IRS2 expression increased apoptosis in these cells, suggesting that IRS2 can contribute to liver tumors (71). Furthermore, it was shown that IRS2 contributes to increased viability and reduced apoptosis in myeloid cancers harboring the activating mutation of JAK2 (JAK2V61F) by interacting with the mutant JAK2, suggesting that IRS2 can be a target to control this disease (80). These authors proposed that pharmacological inhibition of IRS2 may be useful to complement anticancer therapies by increasing apoptosis in tumor cells.

The phosphorylation of IRS proteins leads to the interaction with several signaling proteins. Among them, the PI-3′ kinase has been shown to play an important role in transmitting anti-apoptotic signals downstream of IRS2. The p85 subunit of the PI-3′ kinase coprecipitates with IRS2 and specific inhibitors of PI-3′ kinase blocked the protection from apoptosis by IL-4 on B cells (61). Similarly, the expression of dominant inhibitory forms of PI-3′ kinase abrogated the anti-apoptotic effect of IL-4 on B cells (92). Other intracellular proteins including Akt and p70S6K have been found to act downstream of IRS proteins/PI-3′ kinase in signaling protection from apoptosis (70, 93). It has also been found that insulin and IGF-1 can prevent apoptosis by an IRS2-dependent pathway that requires PI-3′ kinase and Akt (70). IRS proteins have also been reported to signal protection from apoptosis by PI-3′ kinase-independent pathways (94). Thus, the expression of IRS1, but not IRS2, protected a T cell hybridoma from activation-induced cell death (AICD) by a mechanism independent of PI-3′ kinase (94). In this case, pharmacologic inhibition of PI-3′ kinase did not abrogate the resistance of cells expressing IRS1 to AICD. In fact, the protection from apoptosis was independent of tyrosine phosphorylation and association of IRS1 with PI-3′ kinase. The authors suggested that the protection was mediated through serine residues present in IRS1 but not in IRS2. The molecular pathways activated through IRS proteins can lead to the inhibition of caspase activity (72, 73). Thus, the overexpression of IRS1 and IRS2 in neuroblastoma cells can prevent the insulin-dependent activation of caspase-3 by a PI-3′ kinase-dependent pathway (73). In the absence of IRS2, hepatocytes experience high rate of apoptosis after serum withdrawal by a mechanism involving capasase-3. Restoration of IRS2 in these cells reduced apoptosis by decreasing caspase-3 activity through a PI-3-K/Akt signaling pathway (70). In T cell hybridomas, IRS1 expression protected from apoptosis by delaying and decreasing functional FAS ligand expression after TCR engagement (94).

The fact that the IRS proteins, especially IRS2, play an important role in protection from apoptosis by several cytokines and growth factors make them potential therapeutic targets to treat several diseases. This can be useful in designing treatment strategies for certain cancers as mentioned above but also for inflammatory diseases and diabetes in which IL-4 and insulin play an important role. Several strategies to increase expression of IRS2 with pharmacologic agents are being explored to enhance pancreatic β-cell and endothelial cell survival in the context of Type II diabetes (18, 95). However, our current understanding of the relative roles of IRS1 and IRS2 in mediating and modulating allergic diseases is quite limited.

## DIFFERENTIAL ROLES OF IRS2 IN IL-4- VERSUS IL-13-INDUCED ALLERGIC RESPONSES

### IL-4 Versus IL-13—Why?

In early days, it was thought that IL-4 and IL-13 elicited identical signaling pathways (51), since they share receptor complexes (**Figure 3**). The Type I and Type II receptors consist of IL-4Rα/gamma chain ($\gamma_c$) and IL-4Rα/IL-13Rα1 heterodimers,

respectively (96). IL-4 binds with high affinity to the IL-4Rα inducing interaction with the γc to form a ternary complex termed the Type I receptor (**Figure 3**). Alternatively, the IL-4/IL-4Rα complex can interact with the IL-13Rα1 to form the Type II receptor complex. IL-13 does not bind directly to the IL-4Rα; however, its binding to the IL-13Rα1 stimulates interaction with the IL-4Rα to form a Type II receptor complex containing IL-13 instead of IL-4 (**Figure 3**). It is now appreciated that these three different ternary complexes activate signaling pathways that are similar but not identical to each other.

Since IL-4 has a higher affinity for initial binding to its cognate binding chain, IL-4Rα, than IL-13 has for binding to IL-13Rα1, IL-4 tends to elicit STAT6 phosphorylation at lower concentrations than IL-13 (96). However, comparisons of IL-4- and IL-13-elicited responses *in vitro* demonstrated differential biological activity on dendritic cells and macrophages (97–100). Furthermore, examination of effector functions during allergic responses in mice suggested that each cytokine controlled a different aspect of the inflammatory response. Several groups reported differences in Th2 inflammatory responses in allergic lung inflammation and worm infection models using the IL-4 and IL-13 knockout and transgenic mice (101–105). IL-4 and IL-13 were ascribed different roles in the initiation and effector phases, respectively, of allergic lung inflammation in mouse models. Only IL-4 was able to polarize T-cells to the Th2-phenotype as demonstrated by studies in IL-4-deficient (57, 101, 106) and in IL-13Rα1-deficient mice (107). This inability of IL-13 to induce Th2 polarization is easily explained by a lack of surface IL-13Rα1 expression on mouse T-cells (108, 109). The result of much research concluded that IL-4/Type I signaling elicits some of the characteristic features of allergic lung inflammation, such as eosinophilia, but that IL-13/IL-13Rα1 is required for the effector responses in the airways including airway hyperreactivity and mucus production (103–105, 107, 110).

The expression of genes characteristic of alternatively activated "M2" macrophages also demonstrated differential dependence on Type I versus Type II signaling *in vivo*. The M2 genes, *ArgI* and *Chia*, required IL-13Rα1 (Type II receptor signaling) in response to OVA challenge and intratracheal instillation of IL-13 (110). When IL-4 was intratracheally instilled into the IL-13Rα1-deficient animals, induction of *ArgI*, *Retnla*, and *MglI* was maintained, demonstrating that M2 responses are independent of Type II signaling *in vivo*. We also showed that M2 responses are maintained in mice lacking γc, when Th2 effectors are provided exogenously, establishing that either the Type I or Type II receptors expressed on macrophages are sufficient to drive M2 responses during allergic responses *in vivo* (111). Interestingly, Rothenberg et al. also showed that IL-13Rα1 was required for TGF-β production in response to aeroallergen challenge with *Aspergillus* or house dust mite (112). In a model of *N. brasiliensis* infection, the production of Th2 cytokines was measured in leukocytes elicited by allergic inflammation (113). Th2 cells produced both IL-4 and IL-13 in the lungs but only produced IL-4 in the lymph nodes of infected mice. Furthermore, ILC2s and basophils were major sources of IL-13, but not IL-4, in the infected lung. Although these papers described the differential production of and dependence on IL-4 and IL-13 in many of the phenotypic endpoints of Th2-mediated inflammation to allergen challenge or worm infection, few if any described the upstream signaling differences elicited by the two cytokines.

These complex modes of IL-4 and IL-13 action have great implications in the design of effective allergy therapies (114). Early attempts to suppress allergic responses in humans using a soluble form of IL-4Rα to specifically inhibit IL-4 action (it does not inhibit IL-13) did not meet clinical endpoints likely because IL-13-induced responses were not suppressed, and in addition to positive signaling pathways, IL-4 also stimulates regulatory responses that could limit inflammation, such as the suppression of TNFα production (115). Thus, it is necessary to understand the different signaling responses and downstream effects of these two cytokines to rationally design inhibitors of the IL-4- and/or IL-13-induced responses that could be used as therapeutics for asthma and allergies.

The regulation of the IRS2 pathway downstream of IL-4 signaling was described using RNAi-mediated knockdown of regulators of the TORC pathway (116). Warren et al. demonstrated that p70S6K and GRB10 were TORC1-activated negative feedback regulators of IRS2 activity (**Figure 4**). P70S6K regulated IL-4-induced IRS2 tyrosine phosphorylation by serine phosphorylating IRS-2. GRB10 interacted with IRS2 and with NEDD4.2 and reduced the amount of phosphorylated IRS2, likely by targeting it to the proteasome for degradation. Mice lacking TSC1/2, and the TORC1- and 2-specific proteins, Raptor and Rictor, have also been instrumental in revealing the TORC-mediated negative regulation of the IRS2 pathway (117, 118). Macrophages from Tsc1 knockout mice have increased TORC1 activity, leading to diminished Akt phosphorylation of Ser473, as might be expected when removing a negative regulator. The decrease in this surrogate measure of Akt activity resulted in decreased polarization to the M2 program in the macrophages. In terms of the role of the two TORC complexes in regulating the IL-4/IRS/Akt pathway and M2 macrophage polarization, macrophages from Rictor-deficient animals showed diminished Akt Ser 473, NDRG, and FoxO phosphorylation (117, 119, 120). Downstream, M2 polarization was either decreased (119, 120) or unchanged (117). When the TORC1 complex is inhibited with rapamycin, human monocyte-derived macrophages that are polarized to the M2 phenotype undergo apoptosis but not cells polarized to the M1 phenotype (121). Expression of M2 surface markers and other genes was reduced. We also observed a reduction in some but not all IL-4-stimulated M2 genes in a human monocytic cell line following rapamycin treatment (116).

## IL-4 Versus IL-13 Signaling Differences: IRS2

The signaling events initiated by IL-4 or IL-13 binding to their cognate receptors have largely been identified through the use of genetically altered cell lines. As discussed above, the differences in signaling between the IL-4 and IL-13 begin with the activation of different Janus family kinases (11, 24, 49, 122). The IL-4Rα associates with JAK1. The $\gamma_c$ subunit associates with JAK3, and the IL-13Rα1 subunit associates with Tyk2 or in some cases JAK2 (**Figure 3**). Both receptor complexes activate STAT6 through

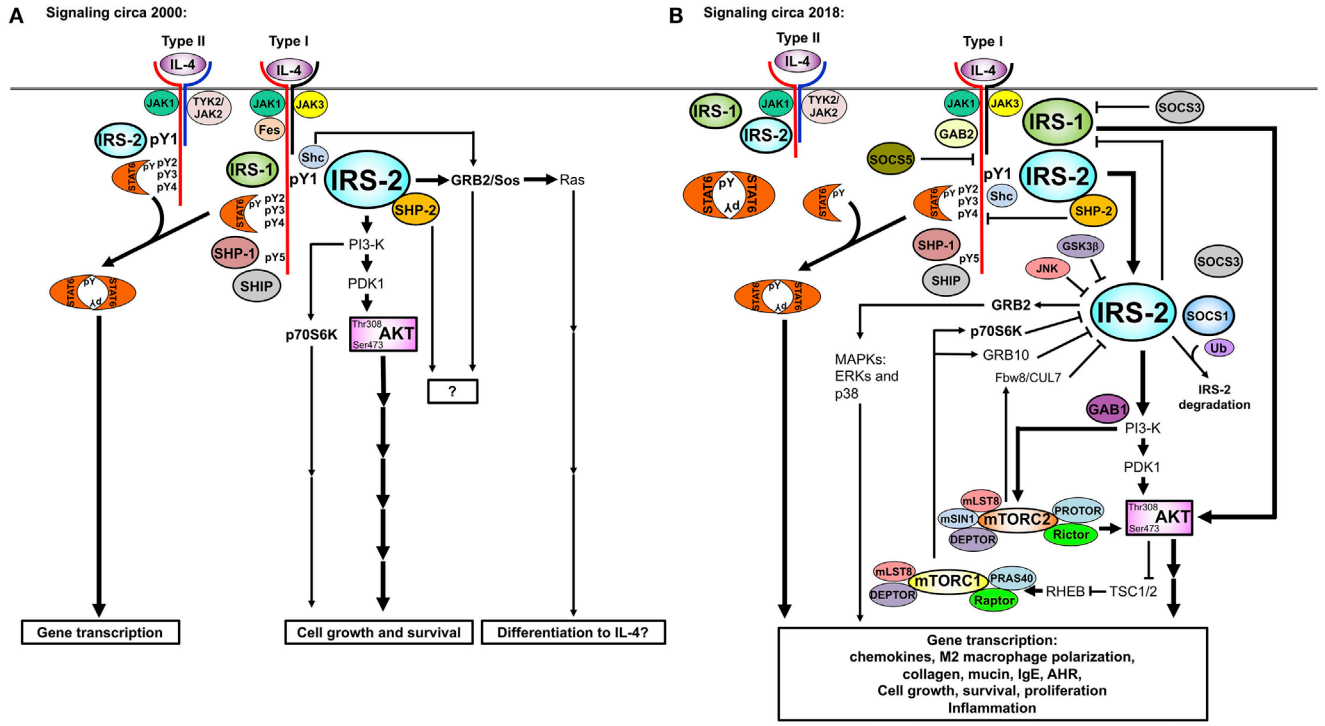


**FIGURE 4** | Historic **(A)** versus updated model **(B)** of IRS signaling. Our understanding of the IL-4-activated IRS signaling pathway has increased substantially between **(A)** 2000 and **(B)** 2018. The involvement of the TOR complexes, SOCS proteins, small adaptor proteins (GRBs and GABs), and other regulatory proteins in negative and positive regulation of IRS2 signaling has been uncovered. The differences in signaling triggered by IL-4 and IL-13 were determined, with IL-4 being a more potent activator of tyrosine phosphorylation of IRS2 than IL-13. Recently, IRS2 has been described as a negative regulator of IRS1, yet many interactions and roles of this pathway remain to be fully understood.

recruitment to the IL-4Rα docking sites and tyrosine phosphorylation by the JAKs (45). Comparisons of IL-4- or IL-13-induced STAT6 phosphorylation in Ramos and A459 cells, which express either only the Type I or Type II receptor complexes, respectively, have revealed interesting differences in potency and kinetics (96). IL-4 stimulated the tyrosine phosphorylation of STAT6 faster and at lower concentrations than IL-13 in all cases, even in the absence of $\gamma_c$ where IL-4-Type II and IL-13-Type II complexes could be compared head-to-head. Furthermore, consistent with historic studies on the exquisite sensitivity of B-cells to IL-4 (123–125), Ramos cells, expressing Type I receptors, exhibited rapid and robust STAT6 phosphorylation at low concentrations of IL-4 that was far superior to responses elicited in A549 cells (Type II receptor complex) (96). This differential responsiveness could be influenced by the relative density of the receptor chains (IL-4Rα, γc, and IL-13Rα1) and by site-directed mutagenesis of the cytokines themselves (126).

While we identified differences in potency and kinetics of STAT6 activation among the three ternary complexes, the degree of STAT6 phosphorylation could ultimately reach equality (64). However, we observed differences in activation of the IRS2 pathway that are more persistent. Comparing two monocytic cell lines, Type I and II receptor expressing U937s and Type II receptor expressing THP-1 cells, Heller et al. showed that robust tyrosine phosphorylation of IRS2 was dependent on the $\gamma_c$ (64). Furthermore, bone marrow-derived macrophages (BMMs) lacking the γc exhibited diminished phosphorylation of IRS2 when stimulated with IL-4 while STAT6 phosphorylation was unaffected. Consistently, the Type II receptor is much less efficient at activating the IRS2 pathway even at high concentrations of cytokines that stimulate equivalent phosphorylation of STAT6.

To understand why IL-4 activates the IRS2 pathway more potently than IL-13, we dissected the role of the Type I and Type II IL-4 receptor complexes in triggering signaling. Since the IL-4Rα chain is shared between both complexes, we used human cells deficient in the γc subunit or macrophages from γc-deficient mice, as well as transfected cells expressing chimeric receptor subunits, to determine the role of the γc and IL-13Rα1 in initiating IRS2 signaling (64, 127). The presence of the γc subunit was critical for full activation of IRS2 signaling in response to IL-4 (64). However, to our surprise, it was the extracellular and transmembrane portions of the γc subunit that determined activation of the IRS2 pathway, rather than the cytoplasmic region of the γc subunit (127). We speculate that the extracellular and transmembrane regions assumed an IL-4-specific conformation that is transmitted to the associated JAKs, resulting in optimal IRS2 activation. Further research is needed to completely understand this aspect of IL-4 versus IL-13 signaling.

Once phosphorylated, IRS2 is able to associate with Grb2 and the p85 subunit of PI 3′ kinase and thereby initiates additional signaling pathways (27). We found that IL-4-activated IRS2 coprecipitates with Grb2 *via* a Type I receptor-dependent pathway (64).

IL-13, while able to induce the tyrosine phosphorylation of IRS2, albeit reduced, did not stimulate the coprecipitation of IRS2 with Grb2. These results suggest that the Type I and Type II receptor complexes differentially stimulate the IRS2/Grb2 pathway. The significance of this difference is still unclear. To date, binding partners of the IRS2/Grb2 complex in the setting of IL-4 signaling have not been identified. Classical pathways downstream of Grb2 in the setting of insulin or IGF treatment such as the RAS-MAPK pathway are typically not activated by IL-4 (27, 64). Characterization of this arm of the IL-4 activated IRS2 pathway and it biological significance will require further investigation.

Activation of PI 3′ kinase through the IRS2 adaptor triggers the Akt pathway, independently of STAT6. This signal then activates the TORC1 pathway and increases the activity of downstream serine threonine kinases. Akt activation leads to the progressive degradation of TSC1/2, molecules which inhibit TORC1 and TORC2 activity (117). Enhanced TORC1 then activates GRB10 and p70 S6K. In addition to stimulating positive pathways, TORC1 induces a negative feedback loop which in the insulin signaling pathway leads to serine phosphorylation of IRS1 and reduced insulin receptor signaling (33, 128). In studies by Warren et al., it was shown that the IL-4-activated Akt/TORC1 pathway induced the serine phosphorylation of IRS2, with a decline in tyrosine-phosphorylated IRS2, indicating a reciprocal relationship between the two posttranslational modifications. Serine phosphorylation of IRS2 by p70S6K and association with GRB10 and NEDD4.2 negatively regulated IRS activity likely by targeting it for proteosomal degradation (116). Macrophages lacking TSC1 have low expression of IRS2 and fail to activate the Akt pathway when stimulated with IL-4 (117, 118). This supports the finding that TORC1 activity downmodulates IRS2 expression. SOCS1, induced during IL-4 signaling, also facilitates the ubiquitination and degradation of tyrosine-phosphorylated IRS2 to negatively regulate IRS2 signaling (32). Interestingly, a defect in SOCS1 induction was observed in allergic asthmatics, suggesting that inhibiting IRS2 signaling is protective against asthma. The proteosomal degradation of IRS1 and IRS2 is blocked by interactions with the ERα in a breast cancer cell line (36). Estrogen also enhances the expression and tyrosine phosphorylation of IRS1 in a variety of breast cancer lines (129). Whether such regulation of IRS proteins by estrogen is in some way responsible for the enhanced M2 macrophage polarization and allergic inflammatory response observed in females is not known (130, 131).

## Differential Responses on Allergic Inflammatory Cells

The difference in signaling pathways elicited by IL-4 compared to IL-13 has distinct effects on responses of cells increased in numbers during allergic responses, including M2 macrophages and eosinophils. The ability of IL-4 or IL-13 to increase expression of genes characteristic of M2 macrophage polarization correlated with the amount of tyrosine-phosphorylated IRS2 (64). On average, M2 macrophage gene expression was approximately 30–50% less in IL-13-stimulated cells compared to IL-4-stimulated cells. Even when activation of the STAT6 pathway was maximal in response to either IL-13 or IL-4, the difference in the activation of IRS2 in IL-13-stimulated compared to IL-4-stimulated BMMs resulted in less mRNA and/or protein encoding *Arg1* (Arginase 1), *Retnla* (Found in inflammatory zone 1, FIZZ1), and *Chi3l3* (YM1). Taken together, these data demonstrated that the IRS2 pathway was poorly activated in response to IL-13 and that IL-4 was a more potent inducer of the M2 macrophage polarization program than IL-13. These findings are important *in vivo* in diseases where M2 macrophages and their secreted proteins play a role in pathology or immunity, such as asthma, allergies, worm infection, or cancer. The relative presence of IL-4 or IL-13 in the microenvironment may shape the magnitude of macrophage polarization.

IL-4 and IL-13 also have distinct effects on other immune cells. IL-4 has been shown to act as a chemoattractant for human eosinophils as well as to enhance chemokine-induced movement (132). We showed that IL-4 enhanced eotaxin-1-induced chemotaxis but IL-13 did not (133). This occurred in an IL-4 concentration-dependent manner and enhancement was dependent on expression of the γc subunit and therefore Type I IL-4 receptor signaling. There were signaling differences in mouse eosinophil responses to IL-4 and IL-13. Activation of STAT6 was greater in response to IL-4 compared to IL-13. This is similar to IL-4 responses in macrophages, airway epithelial cells (A549 cell line), and other cell types. Phosphorylation of IRS2 was also greater following IL-4 stimulation but it was not statistically significant. We speculated that STAT6 signaling might synergize with eotaxin-1-/CCR3-induced PI 3′ kinaseγ activation to enhance chemotaxis through cytoskeletal rearrangement, although this remains to be elucidated. Targeting this pathway would be useful in treating $Th2^{hi}$ allergic asthmatics, where eosinophils and Th2 cytokines play a dominant role in this asthma endotype.

# CONTRIBUTION OF IRS PROTEINS TO ALLERGIC RESPONSES

While the STAT6 pathway has been clearly shown to be an important mediator of the majority of allergic responses *in vivo*, the contribution of the IRS pathway to immune responses is not well understood. We found that transgenic overexpression of IRS2 in lymphocytes enhanced IgE production *in vivo*, and increased the amount of IL-5 produced by *in vitro* differentiated $CD4^+$ Th2 cells (134). Consistent with these findings, *in vitro* studies of T-cells isolated from $IRS2^{-/-}$ mice found modestly reduced T-cell proliferation and production of IL-5 by Th2 cells as compared to T-cells from $IRS2^{+/+}$ mice (62).

Mice expressing a mutation in the I4R-motif (IRS-docking site) of the murine IL-4Rα (Y500F) were developed and studied for allergic responses (135). This mutation impaired T cell proliferation but did not affect Th2 cytokine secretion *in vitro*. Surprisingly, it was found that mice expressing the Y500F form of IL-4Rα demonstrated enhanced parameters of allergic inflammation, including IgE production, airway hyperresponsiveness, eosinophilic inflammation, and mucus production, suggesting a significant contribution of this region of the IL-4Rα to inflammation control *in vivo*. While this mutation abrogated activation of the IRS2 pathway, this region of the IL-4Rα is known to act as a docking site for other signaling molecules including IRS1, Shc, FRIP1, p62DOK, and p85β (49, 136).

As discussed above, we showed that IL-4 elicited robust phosphorylation of IRS2 and M2 gene expression in macrophages *in vitro*, while IL-13 induced significantly weaker responses (64). Moreover, IL-4-mediated signaling and gene induction were reduced in macrophages lacking the γc chain and the Type I receptor. Since the PI 3′ kinase and Akt pathways downstream of IRS2 were reported to be important for M2 differentiation (137), we expected that IL-4-mediated M2 activation would be reduced in the absence of IRS2.

Contrary to expectations, stimulation of IRS2$^{-/-}$ macrophages with either IL-4 or IL-13 enhanced expression of *Retnla*, *Chi3l3*, and *Arg1* mRNA, when compared to WT macrophages (17). Moreover, the differential potency of IL-4 and IL-13 for M2 gene expression was still observed in IRS2-deficient cells. Thus, the reduced quantities of M2 transcripts seen in IL-13-stimulated macrophages are not explained by reduced IRS2 phosphorylation. Another surprising finding was that loss of IRS2, an adaptor that links to PI 3′ kinase, led to increased phosphorylation of Akt and S6 under basal or IL-4-treated conditions. It is likely that this enhanced signaling proceeds *via* IRS1, as knockdown of IRS1 in the IRS2-deficient macrophages abrogated the elevated basal and IL-4-induced responses *in vitro*. These studies reveal a previously unappreciated negative feedback loop downstream of IRS2 during IL-4 signaling and suggest that the IRS1 adaptor positively regulates the M2 phenotype, although a definitive role for IRS1 remains to be established. These results are at odds with our previous work showing robust positive relationships between the IL-4-induced tyrosine phosphorylation of IRS2 and enhanced M2 macrophage differentiation (64). It is possible that compensatory mechanisms in the IRS2-deficient mice lead to enhanced involvement of the IRS1 adaptor that does not normally occur. Alternatively, it is possible that without careful analyses of the serine/threonine and tyrosine phosphorylation status of IRS1, the correlations with tyrosine-phosphorylated IRS2 are misleading (33).

Since M2 macrophages have been shown to enhance allergic responses (138–140), we further evaluated the contribution of IRS2 to allergic lung inflammation *in vivo* (17). IRS2$^{+/-}$ and IRS2$^{-/-}$ mice developed enhanced allergic lung inflammation and increased airway and vascular remodeling in comparison to IRS2$^{+/+}$ mice. In the absence of IRS2, there were increased numbers of eosinophils in the airways and lungs of mice in an acute allergen sensitization/challenge model. There was also a striking increase in muscularization of small vessels that was accompanied by increased production of the M2 macrophage protein FIZZ1 by cells surrounding the blood vessels. However, there was no difference in IgE production, Th2 cytokine levels in the bronchoalveolar lavage fluid, or mucus production by airway epithelial cells. These results suggest a novel, critical role for IRS2 in limiting allergic inflammation and pulmonary arterial remodeling induced by a Th2 immune response. A potential contribution of IRS1 in allergic responses has not yet been explored in animal models or humans (141, 142).

Macrophage adoptive transfer experiments demonstrated that the negative regulation of eosinophilic inflammation and pulmonary arterial muscularization by IRS2 was at least in part intrinsic to the macrophage (17). The potential contribution of IRS1 to these *in vivo* responses is not yet known and remains an important area of investigation. These results suggest novel roles for IRS1 and IRS2 in the regulation of allergic lung disease, and present potential therapeutic strategies.

## FUTURE STUDIES

The most recent work advances our understanding of the complex signaling pathways controlling allergic inflammation and paves the way for targeted manipulation of the IL-4/IL-13 pathway in the quest for additional therapeutic interventions against allergic diseases. Since the early characterization of the contribution of 4PS to cell proliferation and survival (**Figure 4A**), many more layers of regulation have been discovered (**Figure 4B**). However, the full impact of these regulatory pathways on the control of biological responses elicited by IL-4 or IL-13 are unclear. The level of complexity of potential positive and negative regulatory circuits calls for a systems engineering approach to fully understand the integration of these pathways.

Substantial progress has been made over the past 28 years in understanding the contribution of 4PS (IRS1 or IRS2) to IL-4- and IL-13-stimulated responses in the context of allergic diseases, however, as noted throughout, there is still much work to be done. Whether IRS2 is mostly a positive or a negative regulator of IL-4- or IL-13-induced responses represents a fascinating paradox. Should therapeutic strategies strive to increase or inhibit IRS2 *via* manipulation of protein expression or serine/threonine or tyrosine phosphorylation? What about IRS1? Targeting strategies are just beginning to be explored and developed in the context of epithelial cancers and Type II diabetes (18, 80, 95). What about allergic disease? On a broad and philosophical note, why does the IL-4/IL-13 system tap into the signaling pathway so critical for insulin signaling and metabolism? Do the IRS proteins play a role in the reported IL-4-induced control of adaptive thermogenesis (120, 143–145)? The search for the answers to these questions will likely engage the imagination and energy of young investigators and lead to the discovery of new and unexpected pathways controlling IL-4- and IL-13-induced responses. Bill would be delighted.

## AUTHOR CONTRIBUTIONS

ADK developed the topic and theme of the perspective, prepared figures, wrote and edited the manuscript. JZ contributed to the writing and editing of the manuscript. NH and AK wrote and edited the manuscript and prepared **Figure 4**.

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REVIEW
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# The Hunt for the Source of Primary Interleukin-4: How We Discovered That Natural Killer T Cells and Basophils Determine T Helper Type 2 Cell Differentiation *In Vivo*

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Interleukin (IL)-4 plays a central role in determining the phenotype of naïve CD4$^+$ T cells by promoting their differentiation into IL-4-producing T helper type 2 (Th2) cells, which are crucial for the induction of allergic inflammation. However, to date, the potential sources of "primary IL-4" *in vivo*, as distinguished from IL-4 produced by Th2 cells, remain unclear. Here, I describe the research I carried out in collaboration with Dr. William E. Paul to identify "primary IL-4"-producing cells and Th2 cell differentiation *in vivo*.

**Keywords: Dr. William E. Paul, T helper type 2 cell differentiation, interleukin-4, interleukin-18, natural killer T cell**

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## INTRODUCTION

In 1986, Coffman and Mosmann proposed the T helper (Th) dichotomy, in which they showed the presence of two different cell subsets, consisting of Th1 and Th2 CD4$^+$ T cell lineages each expressing a definite cytokine profile (1). CD4$^+$ T cells are differentiated into Th1 cells in the presence of interleukin (IL)-12, which primarily produce IFN-γ and IL-2 and are concerned in cell-mediated immune responses. IFN-γ activates macrophages and is extremely efficient in the elimination of intracellular pathogens. While CD4$^+$ T cells are differentiated into Th2 cells in the presence of IL-4 and produce IL-4, IL-5, IL-9, and IL-13 (2, 3), these Th2 cytokines are critical for the development of allergic diseases and the elimination of helminth infections by the induction of IgE synthesis, the activation of basophils and mast cells, and the recruitment of eosinophils. The theory of a Th1/Th2 balance presented the base for understanding the mechanisms of immune responses and has been generally established as a paradigm of the immune system for over 30 years.

It is well established that the differentiation of naïve CD4$^+$ T cells into Th1 or Th2 cells requires three signals: (1) T cell receptor (TCR) triggering through antigen recognition by MHC class II molecules; (2) augmentation of TCR signaling *via* co-stimulatory molecules, such as CD80 and/or CD86 and CD28; and (3) an appropriate cytokine, e.g., IL-12 for Th1 cell (4, 5) and IL-4 for Th2 cell differentiation (6, 7). In the early 1990s, the *in vivo* source of IL-12, essential for Th1 cell differentiation, was revealed as macrophages or dendritic cells (DCs) in response to pathogens (4, 8). By contrast, potential *in vivo* sources of IL-4, essential for Th2 cell differentiation, remained unclear. Therefore, Dr. Paul gave me a mission to identify sources of IL-4 that promote the differentiation of naïve CD4$^+$ T cells into IL-4 producers. This type of IL-4 was designated as "primary IL-4" to distinguish it from Th2 cell-producing IL-4 (**Figure 1**).

We first discovered a specific subpopulation of helper T cells, CD4$^+$NK1.1$^+$ T cells, which promptly produce significant amounts of IL-4 upon stimulation *in vivo* (9). Next, we showed the property

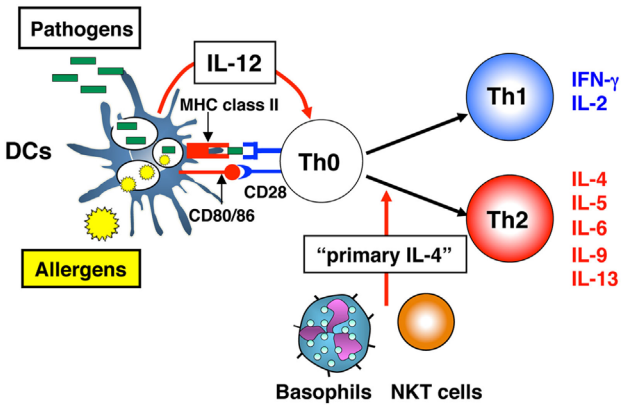


**FIGURE 1** | Differentiation of naïve CD4+ T cells into T helper (Th)1 or Th2 cells. Differentiation of naïve CD4+ T cells into Th1 or Th2 cells requires three signals: (1) T cell receptor (TCR) triggering through peptide-antigen recognition in the context of MHC class II molecules; (2) augmentation of TCR signaling *via* CD80 and/or CD86 and CD28 co-stimulatory molecules; and (3) an appropriate cytokine, interleukin (IL)-12 for Th1 cell differentiation and IL-4 for Th2 cell differentiation. For Th1 cell differentiation, which develops in response to viral and bacterial pathogens, dendritic cells (DCs) function as antigen-presenting cells and provide all three signals. For Th2 cell differentiation, which develops in response to an allergen, DCs cannot provide all three required signals, because of the lack of "primary IL-4," the cytokine essential for Th2 cell differentiation. Cells, such as natural killer T (NKT) cells or basophils are candidate "primary IL-4"-producing cells.

of basophils as "primary IL-4"-producing cells (10). Finally, we revealed that basophils have dual functions as "primary IL-4"-producing cells and as antigen-presenting cells (APCs) which preferentially induce Th2 cells *in vivo* and *in vitro* (11). In this review, I describe the story of research to identify "primary IL-4"-producing cells and Th2 cell differentiation in collaboration with Dr. William E. Paul.

## CD4+NK1.1+ T CELLS ARE A SOURCE OF IL-4 THAT PROMOTES THE DIFFERENTIATION OF NAÏVE CD4+ T CELLS INTO Th2 CELLS

In 1994, Dr. Paul and I showed that almost all amounts of IL-4 produced within 30–90 min after an injection of antibody against anti-CD3 into mice were from an unexpected population of CD4+ T cells that express receptors of the NK lineage, NK1.1, on their surface (9). These CD4+NK1.1+ T cells are somewhat small in the spleen (~1% of splenic cells) and have a specific TCR expression of Vα14 and Vβ8.2, which are specific for MHC class I-like molecules CD1. Today, these cells are termed natural killer T (NKT) cells (12, 13).

Interestingly, the development of NKT cells was markedly impaired in β2-microglobulin deficient ($\beta2M^{-/-}$) mice (14). This is in keeping with the association of β2-microglobulin with CD1. Indeed, splenic cells from $\beta2M^{-/-}$ mice produced little or no IL-4 in response to *in vivo* treatment with anti-CD3 antibody (15). Furthermore, $\beta2M^{-/-}$ mice impaired the presence of IL-4-producing cells 5 days after an injection of goat anti-mouse IgD antibody and produced minimal or no IgE in response to this stimulation. Furthermore, the ability of irradiated $\beta2M^{-/-}$ mice to produce IgE in response to an *in vivo* challenge with anti-IgD antibody can be restored by transferring purified populations of CD4+NK1.1+ thymocytes and T cell-depleted splenic cells from normal mice (15). These results show that the production of IgE depends upon NKT cells, probably because NKT cells can rapidly produce "primary IL-4," which sequentially prime naïve CD4+ T cells to differentiate into IL-4-producing Th2 cells.

SJL mice have a defect in IgE production to a variety of stimulants (16, 17). To reveal the possibility that their defect might be due to a lack of splenic NKT cells, SJL mice were *in vivo* challenged with anti-IgD antibody. As a result, SJL mice had defects in IgE production and IL-4-producing cells in response to this treatment. By contrast, similarly, anti-IgD-treated BALB/c and C57BL/6 mice made substantial amounts of IgE and induced IL-4-producing Th2 cells. In addition, *in vivo* treatment of SJL mice with anti-CD3 antibody also failed to produce "primary IL-4" (18).

These results suggest that the defect in IL-4 and IgE production in two strains of mice—$\beta2M^{-/-}$ mice and SJL mice—was associated with, and might be caused by, an absence of the NKT cells. However, we observed that in response to certain stimulant, $\beta2M^{-/-}$ mice produced IgE. These mice immunized with ovalbumin (OVA) and alum-induced IgE production and IL-4-producing cells (TY and WEP, unpublished work). This may be explained by the production of "primary IL-4" by cell types other than NKT cells.

When Dr. Paul and I published these attractive data, we considered several possibilities when answering the question, "How do NKT cells contribute to Th2 cell differentiation *in vivo*?" as described below. First, peptides derived from allergens or Th2-inducing pathogens, such as helminths, may connect to CD1 molecule and form epitopes recognized by NKT cell receptors. The second possibility is that APCs that interrelate with allergens or Th2-inducing pathogens may regulate the expression level of CD1 or co-stimulatory molecules on their surface. The third possibility is that NKT cells may receive a robust stimulus through the interaction of their receptors with CD1 expressed on the organs such as skin, respiratory tract, and gut. If naïve CD4+ T cells encounter antigens in these organs, they are initiated by "primary IL-4" and differentiated into Th2 cells.

## NKT CELLS RESPOND TO IL-18 TO PRODUCE IL-4 THAT PROMOTES NAÏVE CD4+ T CELLS TO DIFFERENTIATE INTO Th2 CELLS

### IL-18 Induction of IgE: Dependence on CD4+ T Cells and IL-4

In 1995, when I returned to Japan, a new cytokine IL-18 was discovered and cloned at Hyogo College of Medicine (19). IL-18, an IL-1-like cytokine that requires cleavage by caspase-1 to become active form, was originally recognized as a factor that enhanced

IFN-γ production by Th1 cells in the presence of antigen plus IL-12 (19, 20). However, our later studies and those of others revealed that without IL-12 stimulation, IL-18 promotes Th2 cytokine production by $CD4^+$ T cells, basophils, and mast cells (21–25). With IL-3, IL-18 stimulates basophils and mast cells to induce IL-4, IL-9, and IL-13 production even without cross-linkage of FcεRI (21). Naïve $CD4^+$ T cells cultured with IL-2 and IL-18 without engagement of TCR for 4 days produced moderate and significant amounts of IL-4 and IL-13, respectively (23). Additional stimulation with antibodies against CD3 and CD28 increased their capacity to produce IL-4 and IL-13. Moreover, these activated T cells were differentiated into Th2 cells *in vitro*, while naïve $CD4^+$ T cells cultured with the same protocol, but with additional neutralizing antibody to IL-4, were differentiated into Th1 cells, not Th2 cells. These results suggested that IL-18 has the potential to develop Th2 cells in an IL-4-dependent manner (23).

We also demonstrated that in addition to IL-4 production, naïve $CD4^+$ T cells stimulated with IL-2 and IL-18 for 4 days upregulated CD40 ligand (CD40L) and induced B cells to secrete IgE *in vitro* (23). Consistent with these findings, the daily injection of IL-18 into mice induced a significant, dose-dependent increase in serum IgE levels *in vivo* in an IL-4-dependent fashion (23, 24). In addition, transgenic mice overexpressing human caspase-1 in keratinocytes, which have significant increased serum levels of mature IL-18, spontaneously develop atopic dermatitis with high serum levels of IgE. This IgE response disappeared in caspase-1 Tg mice lacking IL-18 or STAT6, a crucial intracellular element for IL-4-signaling pathway, indicating that IL-18- and IL-4-mediated signaling pathways are contributed to their IgE response (23). These results taken together indicate that IL-18 has the potential to induce Th2 cell differentiation. In these experiments, Dr. Paul and Dr. Nancy Noben-Trauth collaborated with us in the evaluation of IL-18-induced IgE response *in vivo* and showed that it was IL-4-dependent using BALB/c IL-4Rα$^{-/-}$ mice (23).

## IL-18-Stimulated NKT Cells Are the Major Source of IL-4

Although it was clearly demonstrated that $CD4^+$ T cells can respond to IL-18 to produce IL-4 *in vivo* and *in vitro* (23, 24), the subset of $CD4^+$ T cells that responded to IL-18 stimulation *in vivo* by inducing the expression of IL-4 and CD40L remained unidentified. Collaborating with Dr. Paul and Dr. Booki Min, we revealed that NKT cells are the target cells for IL-18 as described below (26). The daily injection of IL-18 resulted in increased serum levels of IgE, IL-4, and IL-13 in normal mice but not in CD1$^{-/-}$ mice lacking NKT cells, because NKT cells are positively selected by MHC class I-like molecules CD1 (12). In addition, compared with conventional $CD4^+$ T cells, NKT cells, strongly positive for the IL-18Rα chain, produced large amounts of Th2 cytokines (IL-4, IL-9, and IL-13) and increased their CD40L expression in response to IL-18 plus IL-2 *in vitro* without TCR engagement. Moreover, IL-18- and IL-2-stimulated NKT cells induced *in vitro* IgE isotype switching in B cells. By contrast, MHC class II$^{-/-}$ mice, which lack conventional $CD4^+$ T cells but have NKT cells, failed to produce IgE in response to IL-18 treatment, indicating that conventional $CD4^+$ T cells are important for IL-18-induced IgE production. Actually, these mice, reconstituted with conventional $CD4^+$ T cells from wild type but not from IL-4$^{-/-}$ mice, produced IgE. Thus, these results demonstrated that NKT cells are an essential subset of $CD4^+$ T cells responding to IL-18 by inducing IL-4 production and CD40L expression *in vivo*, and IL-4-producing conventional $CD4^+$ T cells are required for IgE production by B cells together with NKT cells (**Figure 2**).

Natural killer T cells rapidly produce IL-4 after stimulation of the TCR with anti-CD3 antibody (9). Furthermore, without TCR engagement, NKT cells produce a variety of Th2 cytokines including IL-4 in response to IL-18, which has the potential to initiate Th2 cell development and IgE production (26). Therefore, our original work with Dr. Paul on the roles of NKT cells in Th2 cell development at the National Institutes of Health in the USA (9) connected to the discovery of the contribution of NKT cells for IL-18-driven Th2 cell development in Japan (23, 26). Throughout these experiments, Dr. Paul gave us a great deal of support and engaged with us in helpful discussions.

## RECENT STUDIES OF NKT CELLS AS IL-4-PRODUCING CELLS

NK1.1 has been considered to be a marker of NKT cell; however, it is neither expressed in BALB/c mice. Thus, instead of surface markers, recent study performed intracellular staining for transcription factors recognized consistently in different mouse strains. According to the combination of transcription factor (T-bet, GATA-3, and ROR-γt), NKT cells are separated into three distinctive subsets: NKT1, NKT2, and NKT17 cell, analogous to the nomenclature of Th lineage (Th1/Th2/Th17) (27). Lee et al. demonstrated that NKT2 cells highly expressed Th2-specific transcription factor GATA-3, while NKT1 cells expressed a high level of T-bet with low GATA-3. Notably, upon stimulation with PMA plus ionomycin, thymic NKT1, NKT2, and NKT17 cells produced IFN-γ, IL-4, and IL-17, respectively.

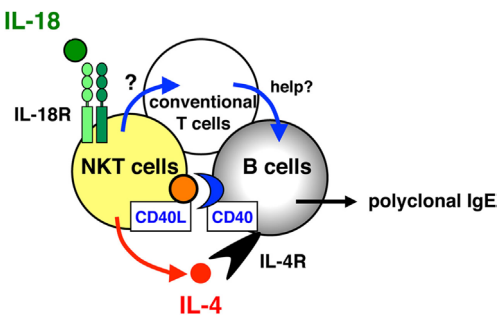


**FIGURE 2** | Role of natural killer T (NKT) cells and conventional T cells for interleukin (IL)-18-induced IgE production *in vivo*. NKT cells, strongly positive for the IL-18Rα chain, are a critical subset of $CD4^+$ T cells that respond to IL-18 by the expression of IL-4 and CD40 ligand (CD40L) *in vivo*, and conventional $CD4^+$ T cells act as helper T cells together with NKT cells in IL-18-induced IgE responses.

Compared with C57BL/6 mice, BALB/c mice have the greater abundance of NKT2 cells and secrete large amounts of IL-4 at a steady state. It is well known that C57BL/6 and BALB/c mice are the prototypes of strains dominating Th1 and Th2 responses, respectively. BALB/c mice have higher serum IgE levels than C57BL/6 mice, while BALB/c background CD1d$^{-/-}$ mice lacking NKT cells significantly reduced serum IgE at a steady state (27). Thus, NKT2 cell-derived IL-4 might modify immune responses under normal steady-state conditions, conceivably contributing to Th2 dominance in BALB/c mice.

Very recently, it has been reported that NKT cells might represent the early source of IL-4 for the initiation of antiviral B cell immunity (28). B cells are essential for the defense against pathogenic infections through the production of pathogen-specific antibodies in germinal centers. In this process, follicular helper T (TfH) cells are known to regulate the initiation of antiviral B cell immunity *via* co-stimulatory molecules and cytokines, such as IFN-γ, IL-4, and IL-21 (29). However, the mechanism by which B cells initially seed germinal center reactions remains unclear. Gaya et al. demonstrated that during influenza infection, there are two waves of IL-4 production: an early wave, mainly produced by NKT cells and restricted to the periphery of B cell follicles, and a late wave, produced by germinal center-resident TfH cells. Furthermore, close interactions between NKT cells and resident macrophages at the follicular through CD1d are necessary to induce early IL-4 production by NKT cells by 3 days after infection. Interestingly, this early IL-4 production by NKT cells was significantly reduced in IL-18R$^{-/-}$ mice, suggesting that IL-18 enhances IL-4 secretion by NKT cells as we reported previously (26). Indeed, they detected a strong accumulation of IL-18 in both subcapsular sinus and medullar macrophages on day 2 of influenza infection, suggesting that these resident macrophages are a source of IL-18. Therefore, early IL-4 production by IL-18-stimulated NKT cells might contribute to the initiation of antiviral B cell immunity.

## BASOPHILS ARE "PRIMARY IL-4"-PRODUCING CELLS

Before serial experiments with NKT cells for Th2 cell development *in vivo*, Dr. Paul had an idea that "primary IL-4"-producing cells might be activated T cells themselves or FcεRI$^{+}$ cells, cells with the morphology of basophils (30, 31). However, neither of these cells appeared ideally suitable to be a physiological source of "primary IL-4" for Th2 cell differentiation. Specifically, the main problem with the theory that basophils might be "primary IL-4"-producing cells is that the only established physiological pathway through which these cells are stimulated to produce IL-4 is by the cross-linkage of FcεRI. In other words, basophil-IL-4 production is dependent upon established Th2 responses of IgE production. However, several studies revealed that basophils might be "primary IL-4"-producing cells for Th2 cell differentiation as described below.

We revealed that without FcεRI cross-linkage, IL-18 stimulated basophils and mast cells to produce Th2 cytokines (21). Murine bone-marrow-derived basophils and mast cells express IL-18Rα chain and produce Th2 cytokines (IL-4, IL-6, IL-9, and IL-13) and histamine in response to IL-3 plus IL-18 stimulation (21). In addition, murine basophils and mast cells express ST2, the receptor for IL-33, a member of the IL-1 family (10). Like IL-18, IL-33 stimulates basophils and mast cells to produce Th2 cytokines without FcεRI cross-linkage. Notably, basophils but not mast cells produce IL-4 in response to IL-3 plus IL-18 or IL-3 plus IL-33 (10).

Proteases secreted from helminths and protease allergens from house dust mites can also induce Th2 cytokines (IL-4, IL-5, and IL-13) from human basophils purified from peripheral blood. Protease inhibitors blocked the production of these Th2 cytokines, suggesting that proteolytic antigens can directly activate basophils (32). Moreover, a cysteine protease allergen papain significantly induced the expression of Th2 cytokines and TSLP in murine basophils (33). Although the receptor or sensors that recognize proteases from allergens and helminths on basophils remain unknown, the downstream signaling pathway activated by papain in basophils was recently characterized (34).

Human basophils express Toll-like receptor (TLR) 2 and produce Th2 cytokines when stimulated with several TLR2-specific ligands (35). We reported that murine basophils selectively express TLR1, 2, 4, and 6 and produce Th2 cytokines in response to IL-3 plus peptidoglycan or IL-3 plus lipopolysaccharide *via* TLR2 or TLR4, respectively (11). It is well known that some infectious conditions induce allergic inflammatory responses. Thus, pathogen-induced Th2 cytokine production from basophils *via* TLRs may contribute to the onset of allergic diseases.

Basophils produce IL-4 significantly and promptly in response to various stimuli, such as IL-18, IL-33, proteases, and TLR ligands, making them a potential candidate for the source of "primary IL-4." Indeed, Min and colleagues reported that in the presence of DCs and antigen, basophils initiated Th2 cell differentiation *in vitro* (36). They showed that naïve CD4$^{+}$ T cells could be differentiated into Th2 cells if they were stimulated with antigen in the presence of basophils and DCs without additional IL-4 (36). Basophil-mediated Th2 cell differentiation was mainly mediated by the IL-4 produced by basophils, because Th2 cell differentiation was not detected when IL-4-deficient basophils were used. In addition, Min and colleagues showed that, at least *in vitro*, the Th2-promoting capacity of basophils was in part due to a direct cell–cell contact with CD4$^{+}$ T cells (36). This led to the later finding that MHC class II expressing basophils functions as APCs, as described below. Nevertheless, their studies clearly provided a proof of principle that basophils can promote Th2 cell differentiation in the presence of DCs and antigen *via* basophil-derived "primary IL-4."

Sokol et al. revealed that basophils are crucial for Th2 cell differentiation in response to papain *in vivo* (33). The immunization of mice with papain alone induced significant Th2 responses, Th2 cytokine production in lymph nodes, and serum papain-specific IgE. Most extraordinarily, basophils quickly migrated into T cell zones of the draining lymph nodes and produced IL-4, 3 days after the *in vivo* injection of papain (33). Taken together,

these results show that "primary IL-4" produced by basophils is essential for Th2 cell differentiation. In this setting, it was initially considered that DCs functioned as APCs and induced Th2 cell differentiation in collaboration with basophil-derived "primary IL-4."

## BASOPHILS HAVE DUAL FUNCTIONS AS "PRIMARY IL-4"-PRODUCING CELLS AND AS APCs THAT PREFERENTIALLY INDUCE Th2 CELL DIFFERENTIATION

In 2009, three independent groups, including ours, published studies showing that besides the function of basophils as "primary IL-4"-producing cells, basophils have the function of APCs to preferentially induce Th2 cell differentiation both *in vitro* and *in vivo* (11, 37, 38). Murine basophils express MHC class II and co-stimulatory molecules (CD80 and CD86). Thus, basophils store all three characters required of Th2-promoting APCs, that is, the expression of MHC class II and co-stimulatory molecules, and the production of "primary IL-4" (**Figure 3**). We showed that basophils also expressed the lymph node-homing molecule CD62L, indicating their potential to migrate to lymph nodes to initiate T cell responses *in vivo* (11). Importantly, human cord blood-derived immature basophils ($CD203c^+c\text{-}Kit^-$) expressed human leukocyte antigen (HLA)-DR (~19%). Furthermore, human peripheral blood-derived mature basophils expressed HLA-DR after *in vitro* culture with IL-3 for 24 h (11). Thus, the expression of MHC class II on basophils is not specific for mouse.

Splenic basophils from gastrointestinal helminth (*Strongyloides venezuelensis*)-infected mice significantly induced naïve $CD4^+$ T cells to develop into Th2 cells without exogenous IL-4. Furthermore, in the absence of DCs, bone-marrow-derived basophils strongly induced naïve OVA-specific $CD4^+$ T cells to differentiate into Th2 cells *in vitro* in the presence of OVA peptide ($OVA_{323-339}$) without additional IL-4. By contrast, splenic DCs induced Th2 cell differentiation only in the presence of IL-4. Additional IL-4 stimulation moderately increased the capability of basophils to induce Th2 cells, whereas basophils from $IL\text{-}4^{-/-}$ mice failed to induce Th2 cells without additional IL-4 (11). From these results, we conclude that endogenous IL-4 from basophils is indispensable for the differentiation of naïve $CD4^+$ T cells toward Th2 cells.

Other groups also demonstrated that basophils expressed MHC class II and promoted the MHC class II-dependent Th2 cell differentiation *in vitro* without additional IL-4 (37, 38). Sokol et al. showed that Th2 cell differentiation was increased in the presence of papain, which stimulated basophils to increase the expression of MHC class II and the production of IL-4 (37), indicating that protease allergen activated basophils to augment their presentation of allergen to $CD4^+$ T cells.

Do basophils increase their potential to act as APCs when stimulated with antigen and antigen-specific IgE? Basophils pulsed with a low dose (6.2 μg/ml) or a high dose (100 μg/ml) of 2,4-dinitrophenyl (DNP)-conjugated OVA protein induced Th2 cells moderately or strongly, respectively. The addition of anti-DNP IgE to this culture, representative IgE–FcεRI cross-linkage, significantly increased Th2 cell differentiation even with a low dose (6.2 μg/ml) of OVA protein (11). These results indicated that $FcεRI^+$ basophils might catch up low doses of antigen that are sufficient to augment antigen-specific Th2 cell differentiation in an IgE-dependent manner (**Figure 3**).

To reveal how basophils contribute to the development and the augmentation of *in vivo* Th2 cell–IgE responses, naïve mice or basophil-depleted mice were intravenously injected with the complex of DNP–OVA and anti-DNP IgE. This treatment preferentially induced OVA-specific Th2 cell differentiation in the spleens and OVA-specific serum IgG1 in naïve mice, whereas

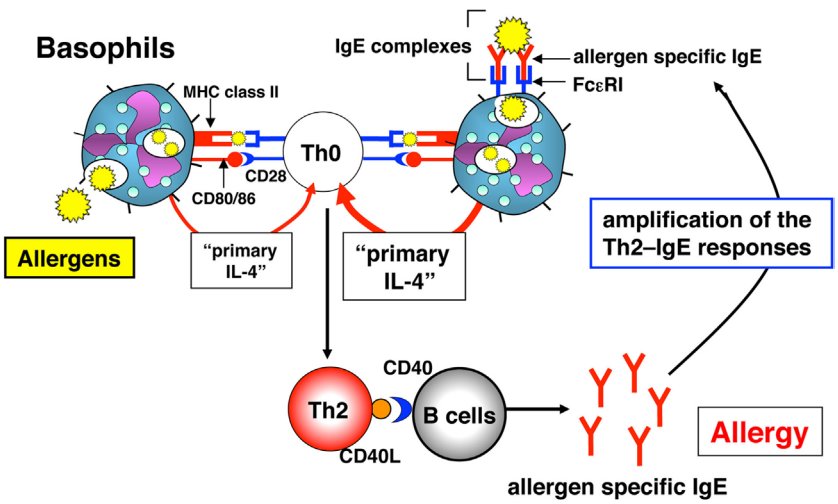


**FIGURE 3** | The role of basophils in the induction and amplification of T helper (Th)2 cell responses. Basophils initially induce Th2 cells *in vivo via* "primary interleukin (IL)-4" production and the presentation of complexes of allergen peptide plus MHC class II to $CD4^+$ T cells. These activated Th2 cells produce IL-4 and express CD40 ligand (CD40L), which, in combination, induce B cells to proliferate and produce allergen-specific IgE *in vivo*. Immune complexes of allergen and allergen-specific IgE "preferentially" augment the development of allergen-specific Th2 responses in an endogenous basophil-dependent manner.

these Th2 responses were significantly diminished in basophil-depleted mice (11). These results clearly demonstrated that basophils contribute to the development and the augmentation of antigen-specific Th2 cells *in vivo* by taking up the complex of antigen and antigen-specific IgE, presenting antigen peptide along with MHC class II and producing large amounts of IL-4 (**Figure 3**).

## CONTROVERSIES IN THIS FIELD OF BASOPHILS

It was clearly demonstrated that basophils are "primary IL-4"-producing cells and that basophils have the function as APCs to promote Th2 responses both *in vitro* and *in vivo*. This paradigm shift was greeted with great enthusiasm, but also with objection (3, 39–42).

In 2009, researchers including our group used basophil-depleted mouse models based on antibody-mediated depletion strategies using FcεRIα-specific antibody (MAR-1). Some controversial evidences demonstrated that basophiles were not essential for Th2 differentiation and IgE production *in vivo* analysis using a basophil-specific deletion system. Ohnmacht et al. generated transgenic mice that express the Cre recombinase under control of regulatory elements for the mast cell protease 8 (*Mcpt8*) gene, which is expressed in basophils (43). More than 90% of basophils were constitutively deleted in *Mcpt8Cre* mice. They clearly demonstrated that papain-induced Th2 cell differentiation depended on DCs and not on basophils. Furthermore, they showed that basophils were not required for gastrointestinal helminth (*Nippostrongylus brasiliensis*)-induced type 2 immunity (43). Sawaguchi et al. established diphtheria toxin-based conditional basophil deletion mice, Bas-TRECK mice (44). OVA in alum-immunized Bas-TRECK mice showed equivalent serum OVA-specific IgE levels as control mice, indicating that basophils are dispensable for the development of a systemic IgE response (44).

Some groups have demonstrated that basophils had no functions as APCs and that DCs were essential APCs to promote Th2 responses in mouse models of inhaled house dust mite allergen (45) or helminthic infection (46). In addition, we and others demonstrated that basophils contribute to the cutaneously induced Th2 cell differentiation (47–50). Otsuka et al. reported a possible explanation for the controversial functions of basophils as APCs (48). In their model, basophils functioned as APCs and sufficiently initiated Th2 responses if the antigen was a hapten or a peptide. Other groups demonstrated the collaboration between basophils and DCs, where basophils promoted Th2 cell differentiation in combination with DCs as "primary IL-4"-producing cells (47, 49). The epicutaneous application of a vitamin D analog (49) or the subcutaneous injection of papain (47) induced the local production of TSLP in skin, which activated DCs to upregulate OX40L and migrate into the draining lymph node. Our group demonstrated that both basophils and TSLP had crucial roles in the development of cutaneously sensitized food allergy by the induction of Th2 responses (50). In that study, basophil-depleted or TSLP receptor-deficient mice were completely defective for Th2 responses against sensitized antigen. Basophils in the regional lymph nodes from mice epicutaneously sensitized with OVA produced more IL-4 than those from naïve mice. As a result, in the case of epicutaneously sensitized protein antigens, basophils were essential for the development of Th2 responses, as they are indispensable producers of "primary IL-4." Therefore, skin is an exceptional organ where basophils have essential roles in the initiation and the development of Th2 responses. Furthermore, we demonstrated that basophil–TSLP pathways in skin were indispensable for the production of antigen-specific IgE and the development of gastrointestinal food allergy (50). Taken together, these reports demonstrated that basophils might contribute to Th2 responses in an organ-dependent manner, and skin could be a unique organ that needs basophils to induce most favorable Th2 responses. Nevertheless, the function of basophils as APCs in the development of Th2 responses is still highly controversial.

Recently, Miyake et al. revealed the functional relevance of basophils in Th2 cell differentiation (51). They demonstrated that basophils acquired complexes of peptide and MHC class II from DCs *via* trogocytosis in a cell-contact-dependent manner both *in vitro* and *in vivo*. That these peptide–MHC class II containing basophils might function as APCs and induce the differentiation of naïve $CD4^+$ T cells into Th2 cells is very interesting. However, without any other APCs including DCs, we demonstrated that basophils strongly induced the differentiation of naïve OVA-specific $CD4^+$ T into OVA-specific Th2 cells *in vitro* in the presence of OVA protein instead of OVA peptide ($OVA_{323–339}$) without exogenous IL-4 (11). Thus, basophils can process OVA protein into $OVA_{323–339}$ peptide and display peptide fragments together with MHC class II and to produce "primary IL-4."

From the beginning of our basophil-APC experiments in 2006, Dr. Paul gave us critical suggestions and a great deal of support. After our publication (11), Dr. Paul mentioned in his review article (3) as described below "Basophils have important roles in the initiation of Th2 cell responses by producing Th2-associated cytokines in response to allergen or helminth-derived products. Basophils are also involved in the initiation of some Th2 cell responses by serving as APCs. However, the differential requirements for basophils or DCs as APCs for the induction of Th2 cell responses seem to depend on the nature of the antigens or helminths and/or the particular adjuvant used."

## CONCLUDING REMARKS

Although IL-4 is essential for both *in vitro* and *in vivo* Th2 cell differentiation, the IL-4/IL-4R/STAT6-signaling pathway is not crucial in some instances of *in vivo* Th2 cell differentiation (52, 53). In addition to IL-4, other pathways such as GATA-3 and GATA5 (54, 55), and cytokines such as TSLP, IL-25, and IL-33 (3, 56, 57) have crucial roles in the induction of Th2 cell differentiation *in vivo*. A recent study suggested that IL-33 plays an important role in the induction and the augmentation of Th2 responses. Halim et al. demonstrated the role of group 2 innate lymphoid cells (ILC2s) in the differentiation of naïve $CD4^+$ T cells into Th2 cells in the lung in response to the protease

allergen papain (57). ILC2s, innate counterparts of adaptive Th2 cells, are activated by IL-33 from allergen-stimulated lung epithelial cells and produce large amounts of IL-13 (58). They showed that although IL-4 was dispensable for papain-induced Th2 cell differentiation, IL-13 derived from ILC2 was crucial since it induced the recruitment of activated CD40$^+$ lung DCs into the draining lymph nodes where they promoted naïve CD4$^+$ T cells to differentiate into Th2 cells (57).

In this review, I have described the long search for "primary IL-4"-producing cells and Th2 cell differentiation carried out in collaboration with Dr. Paul. We identified the key cells (NKT cells and basophils) and molecule (IL-18) involved in priming and developing *in vivo* Th2 responses. However, we will face a major challenge in trying to understand the detailed relations that shape "the nature of the immune response."

In 1997, when we established a new Institute at the Hyogo College of Medicine in commemoration of the discovery of IL-18 (19), Dr. Paul named it the "Institute for Advanced Medical Sciences" and provided a good acronym "IAMS." The phrase for the celebration of the initiation of the Institute: "Organizing scientists to reveal the secrets of nature for the good of man" reminds us that each of us has the responsibility to reveal "the secrets of nature for the good of man" by following Dr. Paul's spirit.

## AUTHOR CONTRIBUTIONS

TY performed the experiments and wrote the manuscript.

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MINI REVIEW
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# Tuning the Cytokine Responses: An Update on Interleukin (IL)-4 and IL-13 Receptor Complexes

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Interleukin (IL)-4 and IL-13 are related cytokines that regulate many aspects of allergic inflammation. They play important roles in regulating the responses of lymphocytes, myeloid cells, and non-hematopoietic cells. In T-cells, IL-4 induces the differentiation of naïve CD4 T cells into Th2 cells, in B cells, IL-4 drives the immunoglobulin (Ig) class switch to IgG1 and IgE, and in macrophages, IL-4 and IL-13 induce alternative macrophage activation. This review gives a short insight into the functional formation of these cytokine receptors. I will discuss both the binding kinetics of ligand/receptor interactions and the expression of the receptor chains for these cytokines in various cell types; both of which are crucial factors in explaining the efficiency by which these cytokines induce intracellular signaling and gene expression. Work initiated in part by William (Bill) E. Paul on IL-4 some 30 years ago has now grown into a major building block of our current understanding of basic immunology and the immune response. This knowledge on IL-4 has growing clinical importance, as therapeutic approaches targeting the cytokine and its signal transduction are becoming a part of the clinical practice in treating allergic diseases. Just by reading the reference list of this short review, one can appreciate the enormous input Bill has had on shaping our understanding of the pathophysiology of allergic inflammation and in particular the role of IL-4 in this process.

**Keywords: interleukin-4, signal transduction, STAT6, interleukin-4 receptor, cytokine signaling, allergic inflammation**

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## INTRODUCTION

Allergic inflammation is an inappropriately controlled inflammatory response with characteristic hallmarks of eosinophilia, elevated immunoglobulin (Ig)E-levels, increased mucus production, and typical cytokine/chemokine expression. Clinically, these basic pathophysiological mechanisms result in symptoms varying from mild skin rash (atopic dermatitis) and runny nose (allergic rhinitis) to life-threatening problems in breathing (allergic asthma). This inflammatory process from the very initiation is critically regulated by cytokines and chemokines. The cytokines regulate cellular responses on transcriptional level, while chemokines play a role in recruiting inflammatory cells to the sites on inflammation. One of the central cytokines regulating allergic inflammation is interleukin (IL)-4 and since its cloning, efforts targeting IL-4 have been made to decrease IL-4-induced inflammation. In part, these efforts have been slowed down by the receptor of IL-4, which is ubiquitously expressed and easily saturated by the ligand. In this minireview, I briefly discuss the receptor system of IL-4 that is also shared by IL-13, how it elicits signaling, and how it has been recently therapeutically

targeted. I also highlight the enormous input of Bill Paul in this field; learning the story of IL-4 is not only about IL-4 but has also helped in unfolding more profound biological phenomenon in how T cells can dynamically respond to changes in environment to output an appropriate response.

## IL-4 AND IL-13 PRODUCTION

Interleukin-4 and IL-13 are the signature cytokines of the type II inflammatory response. They are key players in the inflammatory response triggered either by an invading parasite or allergen. The cellular sources of IL-4 and IL-13 have been studied extensively and along with CD4 T cells, basophils, eosinophils, mast cells, and NK T cells, appropriately stimulated ILC2 cells have the ability to produce IL-4 and IL-13 (1–9).

The genomic locus, where IL-4 and IL-13 are produced (along with IL-5), is called the Th2 cytokine locus, which is located on chromosome 5 in humans and on chromosome 11 in mice and is under the control of the locus control region (LCR) of the Rad 50 gene (10, 11). The LCR in CD4 T-cells is indispensable for the production of IL-4 and IL-13 *in vivo* (12). The production of the two cytokines is not identical though: IL-4 production is calcineurin dependent, whereas IL-13 production is only partially dependent on calcineurin (13). Upon the appropriate stimulation of the cells, the LCR of the Th2 cytokine locus is epigenetically modified to allow the access of transcription factors to the DNA and the subsequent transcription of these cytokines. This complex regulation was recently reviewed in detail (10). Interestingly and in line with findings in mice, a polymorphism in the murine equivalent of the DNase I hypersensitive site (RHS)7 in humans affects DNA methylation and gene expression at 5q31 and subsequently IgE levels on a population level (14).

## IL-4 RECEPTOR SYSTEM

When IL-4 or IL-13 is released from T cells, cells carrying the receptors for these cytokines will respond. For IL-4 and IL-13, the unique utilization of the STAT6 transcription factor in the signaling they elicit allows them to execute specific functions on different cell types; IL-4 is the regulator of lymphocyte functions (Th2 differentiation and B-cell IgG1 and IgE class switch), whereas IL-13 is an effector cytokine, regulating smooth cell muscle contraction and mucus production in the airway epithelium, for example, in allergic asthma (15). In addition to IL-4 and IL-13, one report has shown that at least in human cells, thymic stromal lymphopoietin (TSLP) can induce the tyrosine phosphorylation of STAT6 (16), TSLP signaling will be discussed in detail below.

The cytokine-binding receptor chain for IL-4 is IL-4Rα. This receptor chain is widely expressed, most cells carry at least low numbers of this receptor chain. Upon IL-4 binding to IL-4Rα, the IL-4/IL-4Rα-complex will bind a secondary receptor chain, either IL-2Rγc (γc) or IL-13Rα1 (**Figure 1**). The expression of these secondary chains varies among different cell types. In non-hematopoietic cells, γc expression is low or absent, whereas higher amounts of IL-13Rα1 are expressed in these cells. By contrast, lymphocytes express only low levels of IL-13Rα1 and relatively large amounts of γc. Finally, myeloid cells fall in between non-hematopoietic cells and lymphocytes, as they express of both IL-13Rα1 and γc.

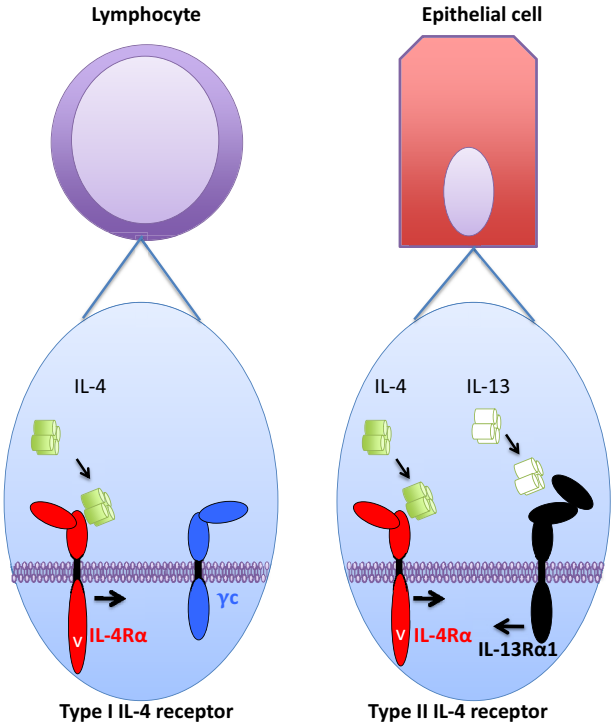


**FIGURE 1** | Type I and type II interleukin (IL)-4 receptor components and cellular distribution. Type I IL-4 receptor is mainly expressed in hematopoietic cells, and specifically in lymphocytes (left part) very little or no expression of type II receptor is observed. In non-hematopoietic cells, such as epithelial cells (right part), very little or no expression of type I IL-4 receptor is observed. Instead, type II IL-4 receptor is readily expressed and subsequently these cells are also responsive to IL-13 that utilizes type II IL-4 receptor, but "drives" it into opposite direction than IL-4. Myeloid cells (not pictured) fall in between these two cell types as they express both type I and type II IL-4 receptors.

Interleukin-4 and IL-13 regulate cellular functions and activate transcriptional machinery *via* cell surface receptors. For IL-4, binding of the cytokine to a single cell surface receptor chain (IL-4Rα) generates a ligand/receptor complex that requires the recruitment of a third receptor chain to form a functional receptor complex. The receptor formed by IL-4/IL-4Rα with γc is a type I IL-4 receptor and the IL-4/IL-4Rα complex binding IL-13Rα1 is a type II IL-4 receptor (17). Thus, based on their tissue distribution, the type I IL-4 receptor is found in lymphocytes and myeloid cells, and the type II IL-4 receptor is expressed in myeloid cells and all non-hematopoietic cells. The binding of IL-4 to IL-4Rα occurs with high affinity (Kd in the order of $10{\cdot}10\ M^{-1}$). This effectively means that at very low concentrations of IL-4 it can maximally occupy the receptor chains at a given cell surface.

It was originally assumed that the secondary recruitment of either γc or IL-13Rα1 into the IL-4/IL-4Rα dimer would occur with substantially lower affinity than the primary binding of IL-4 to IL-4Rα (18, 19). The expression levels of the secondary receptor chain would then become important. As the primary receptor chain for IL-4 is saturated easily, the formation of a

functional receptor complex could be dictated by the availability of the second receptor chain (20). However, the initial binding measurements for the IL-4/IL-4Rα complex binding to γc or IL-13Rα1 were carried out in free solution. Cell membrane-bound γc and IL-13Rα1 behave differently in recruiting the IL-4/IL-4Rα complex under conditions of maximal ligand occupancy (21). While the recruitment of membrane-bound γc is relatively inefficient, the recruitment of IL-13Rα1 takes place roughly with the same efficiency as does the IL-13 driven IL-13/IL-13Rα1 binding to IL-4Rα (21). The authors suggested that early endosomes concentrated the receptor chains underneath the plasma membrane. However, if this is the case, it still remains unclear how IL-4 and IL-13 induce the phosphorylation of STAT6 differently in type I IL-4R-deficient macrophages from different locations, namely, BMDM and peritoneal cavity macrophages (20). If it is not the differential expression of IL-13Rα1 that explains the difference in the cytokine response between these macrophage populations, then a more profound difference in the IL-4Rα-induced STAT6 signaling pathway must be involved which remains uncharacterized. One plausible explanation might be differences in receptor endocytosis between the cells. For IL-13-induced type II IL-4 receptor signaling, IL-13 variants showing decreased IL-4Rα recruitment to the complex indicate that STAT6 signaling is regulated by receptor endocytosis (22). Quite recently, the role of the receptor transmembrane domain in regulating the recruitment of the type II IL-4 receptor has also become appreciated, and the cell type specific actin-dependent membrane microcompartments may participate in dictating the signaling potency of the type II IL-4R (23).

Once completely assembled, the IL-4 receptor complexes will induce intracellular signaling. The binding of IL-4 to the ectodomain of the IL-4Rα and subsequently to γc or IL-13Rα1, induces a conformational change in the intracellular receptor domains allowing the activation of intracellular signaling molecules. The Jak kinases, associated with γc (Jak3), IL-4Rα (Jak1), or IL-13Rα1 (Tyk2, Jak2), will auto- and cross-phosphorylate each other, resulting in their activation and the subsequent tyrosine (Y) phosphorylation of critical Y residues in IL-4Rα chain. Upon phosphorylation, the Y residues in the intracellular domains of IL-4Rα serve as docking sites for SH domains of intracellular signaling molecules (17). STAT6 and IRS molecules, in particular, become activated on these tyrosine residues in response to the activation of the type I IL-4 receptor. By contrast, the type II IL-4 receptor is unable to activate IRS significantly, whereas the activation of STAT6 occurs quite efficiently, which also means that IL-4 (*via* type I IL-4 receptor) activates IRS2 efficiently while IL-13 does not (24). Once activated, STAT6 molecules homodimerize and translocate to the nucleus where they bind specific accessible DNA sequences, for example, on the CD23 promoter in human B-cells and on the arginase1 enhancer in mouse macrophages (25, 26). IRS molecules do not translocate to the nucleus, but rather, they activate signaling pathways independent of STAT6 including PI3K, Akt, PKBE, and mTOR [reviewed in Ref. (27)].

In addition to signaling events that elicit transcriptional changes, pathways that negatively regulate activated signaling pathways are also upregulated by IL-4. Phosphatases, SOCS, and PIAS proteins all participate in the downregulation of the elicited signal, for detailed reviews on these inhibitory mechanisms, see Ref. (28, 29).

## IL-13 RECEPTOR SYSTEM

Like IL-4, IL-13 also has two receptors, but unlike IL-4, IL-13 utilizes two separate binding chains, namely, IL-13Rα1 and IL-13Rα2. Thus, the decision of whether a type I or a type II IL-4 receptor is formed occurs *after* the IL-4/IL-4Rα complex is formed, whereas IL-13 binding *upon* either IL-13Rα1 or IL-13Rα2 determines which receptor IL-13 utilizes. IL-13Rα2 binds IL-13 with higher affinity than IL-13Rα1. The role of IL-13Rα2 in IL-13 biology has been somewhat elusive, and it has been considered merely a decoy receptor that binds free IL-13 strongly, without eliciting signaling, and thus would serve as a "neutralizer" of IL-13, by efficiently internalizing IL-13 from extracellular spaces. Further studies on IL-13Rα2 have shown that the receptor chain is not only a decoy receptor. Indeed, Fichtner-Feigl and colleagues showed a role for IL-13Rα2-mediated signaling that required the cytoplasmic tail of IL-13Rα2 in the production of TGF-β1 providing evidence for IL-13Rα2-mediated signaling (30).

The IL-13Rα1-bound IL-13 "drives" the type II IL-4 receptor into the opposite direction, as does IL-4 (**Figure 1**). Thus, IL-13 binds IL-13Rα1, and the IL-13/IL-13Rα1 complex then recruits IL-4Rα into the functional receptor complex. The fully assembled receptor complex then activates the STAT6 transcription factor, but like IL-4 *via* the type II IL-4 receptor, IL-13 is a poor inducer of IRS activation through this receptor (24). The binding of IL-13 to IL-13Rα1 is relatively inefficient, indicating that once IL-13/IL-13Rα1 binding occurs, the ensuing formation of the functional receptor complex is likely. However, lowering the IL-13/IL-13Rα1-binding capability to IL-4Rα requires a substantial decrease in the second binding step to result in lowered STAT6 activation (22).

## IL-4- AND IL-13-INDUCED SIGNALING: A COMPARISON OF SIGNALING INDUCED BY THE TWO CYTOKINES

Depending on the cell type, IL-4 and IL-13 both can activate STAT6 (**Figure 1**). As IRS2 is only weakly induced by type II IL-4 receptor [and thus IL-13; (24)], intracellular signaling elicited by the two cytokines is somewhat different. By inducing IRS2, IL-4 subsequently activates various pathways including Sos/Ras, PI3K/Akt, PKB/mTOR, or PKC [reviewed in Ref. (31)]. Of these pathways, mTOR has recently been linked to CD4 Th2 cell differentiation as well as alternative macrophage activation these results were recently thoroughly reviewed (32). Unfortunately, experimental therapeutic efforts targeting mTOR in murine allergic disease models have failed (33). Here, it is of note though that mTOR-based approaches target type I IL-4 receptor (i.e., IRS2 signaling), while many disadvantageous IL-4 effects, such as compromised epithelial barrier function, arise from IL-4 signaling *via* type II IL-4 receptor (34).

As pointed out earlier, lymphocytes respond poorly to IL-13. The expression of IL-4Rα (i.e., type I IL-4 receptor) plays thus

a main role in lymphocyte responses to IL-4. The expression of IL-4Rα in naïve lymphocyte is relatively low and *in vitro*, a STAT5-dependent, STAT6-independent signal likely enhances IL-4Rα expression, which then in an autocrine manner, further upregulates IL-4Rα expression (35). Th2 cells then express large amounts of IL-4Rα and are further stimulated *via* IL-4. In case of Th1 or Th17 cells, the lack of IL-4-positive signal inhibits the upregulation of IL-4Rα, but in the case of Th1 cells, for example, the differentiation does not ablate the ability of the cells to respond to IL-4 (36). Interestingly, Th17 cells do express IL-13Rα1 (37).

For ILCs, the expression of IL-4 and IL-13 receptor(s) is still unclear. Several reports have established the ILC2-derived IL-13 acting on target cells *via* type II IL-4 receptor as a mechanism for several physiological functions such as beige fat biogenesis (38) or hepatic fibrosis (39) but if ILC2-derived IL-13 can act on autocrine manner has not been established. Future experiments will also be warranted to reveal if IL-4Rα is differently expressed between ILC subtypes to tune the cells either to IL-4 or IL-13.

## THERAPEUTIC UTILIZATION OF THE IL-4 RECEPTOR SYSTEM

The road for IL-4- and IL-4R-based treatments from bench to bedside has been a long and winding one (40). IL-4 has been considered a therapeutic target for boosting and redirecting T and B cell functions, but the usage of IL-4 itself has been problematic, not least due to the harmful side effects of activating the type II IL-4 receptor in non-hematopoietic cells (34). Furthermore, in mice, IL-4, but not IL-13, induced weight loss and spontaneous erythrophagocytosis (41). Theoretically, in this sense, an IL-4 that could activate only the type I IL-4 receptor but not the type II receptor could be advantageous. Structural studies of human IL-4 receptor complexes (18) indicated that once IL-4 is bound to IL-4Rα, the D-helix of IL-4 faces the secondary receptor chain and forms the interacting surface of IL-4/IL-4Rα to the second chain in question. This opened up opportunities to mutate the structure of the human IL-4 at the D-helix in a way that left the IL-4/IL-4Rα interaction intact but allowed the binding efficiencies of the IL-4/IL-4Rα complexes toward either γc or IL-13Rα1 to be altered. These studies indicated that a 1,000-fold induction in the recruitment of the IL-4/IL-4Rα complex to the secondary chain had surprisingly little effect on the immediate signaling induced by such an IL-4-mutant, as measured by STAT6 activation (42) and similar results were obtained with IL-13Rα1 bound IL-13 mutants with varying abilities to recruit IL-4Rα into the type II IL-4 receptor complex (22). However, in the case of the type I IL-4 receptor, when the availability of the second chain (γc) was decreased with a blocking antibody, the difference between the WT and the type I receptor-specific IL-4 mutant became more evident, suggesting that such IL-4 mutants could be used to redirect IL-4 responses into cells expressing small amounts of second chains for IL-4/IL-4Rα complexes (42).

When considering the harmful effects arising from excess IL-4 and IL-13, in for example allergies, knowledge of the structural and functional characteristics of the IL-4 receptors and their unique signaling *via* STAT6 has been useful in efforts to therapeutically modify IL-4/IL-13 biology. As an example of some therapeutic approaches used are indicated in **Table 1**. A set of monoclonal antibodies for blocking different aspect of the early events of IL-4 and IL-13 signaling are being considered for wider clinical use: dupilumab (43)—a monoclonal blocking antibody for IL-4Rα—lebrikizumab (44), anrukinzumab (45), tralokinumab (46)—blocking antibodies for IL-13—and pascolizumab—a blocking antibody for IL-4 (47) among others. Furthermore, pitrakinra, an IL-4 receptor antagonist that upon binding IL-4Rα, blocks both type I and type II IL-4 receptors has showed initial efficacy in clinical trials (48). The utilization of biological approaches to target IL-4/IL-13 pathways requires an understanding of the pathophysiological process underlying the inflammatory response. The cell type- and tissue-specific distribution of the IL-4/IL-13 receptor components adds to the complexity of the picture and probably in part explains this long and winding road of IL-4R system-based treatments from the initial cloning of the receptor and cytokines to the development of useful clinical applications. Interestingly, STAT6 inhibitor (AS1517499) has shown some potential in inhibiting prostate cancer cell growth [**Table 1**; (49)], which opens new possibilities in targeting the IL-4/IL-13 signaling therapeutically even beyond allergic diseases.

## ANOTHER SHARED CYTOKINE RECEPTOR SYSTEM: IL-7/TSLP

An analogous way of sharing cytokine receptor chains, as seen in the IL-4/IL-13 system, can be found in IL-7/TSLP receptor signaling. In this system, IL-7-bound-IL-7Rα binds γc and thus forms the complete IL-7 receptor, while TSLP binds TSLPR and then recruits IL-7Rα to the complex [reviewed in Ref. (50)]. Thus, theoretically, the IL-7/IL-7Rα/γc complex resembles the type I IL-4 and TSLP/TSLPR/IL-7Rα resembles the type II IL-4 receptor "driven" by TSLP. Furthermore, it is intriguing that TSLPR and γc are closely related structurally, sharing 24% identity to the common γ receptor chain (γc) (51, 52) with certain specific features associated with TSLPR as opposed to other type I cytokine receptors, including the PSxW(S/T) sequence cassette as opposed to WSxWS in the membrane proximal

**TABLE 1** | Examples of various steps interleukin (IL)-4/IL-13 signaling could potentially be targeted.

| Molecule name | Target molecule | Potential | Reference |
|---|---|---|---|
| Dupilumab | IL-4Ra | IL-4- and IL-13-mediated signaling | (43) |
| Pitrakinra | IL-4Ra | IL-4- and IL-13- mediated signaling | (48) |
| Leprikizumab | IL-13 | IL-13-mediated signaling | (44) |
| Anrukinzumab | IL-13 | IL-13-mediated signaling | (45) |
| Tralokinumab | IL-13 | IL-13-mediated signaling | (46) |
| Pascolizumab | IL-4 | IL-4-mediated signaling (trials aborted) | (47) |
| AS1517499 | STAT6 | IL-4- and IL-13-mediated signaling/ transcription/proliferation in prostate cancer cells | (49) |

domain (53). However, this is where the analogy ends, as IL-7/IL-7Rα does not recruit TSLPR, but only γc to the receptor complex. Functionally, it seems that the IL-4/IL-13 receptor is "tuned" for differential purposes than is the IL-7/TSLP system. IL-4Rα is expressed ubiquitously and the second receptor chain (either γc or IL-13Rα1) is also widely distributed. Thus, IL-4 has access to virtually all cell types, and it can saturate receptors at low concentrations due to the efficient primary binding of IL-4 to IL-4Rα. For IL-13, the cytokine concentration required to saturate IL-13Rα1 needs to be higher as the binding efficiency of IL-13 binding to IL-13Rα1 is lower. In line with this, when PBMCs from atopic patients were stimulated with a mite allergen, the cells produced over 20 times more IL-13 than IL-4 (54). The notion of the "effector" function of IL-13 in, for example, parasite expulsion, combined with the known toxicity of IL-4, suggest that the system has evolved in a way that protects peripheral tissues from the toxicity of IL-4 by tuning the receptors in the periphery to be more responsive to IL-13 than to IL-4.

In the IL-7/TSLP system, the differential anatomical expression of the cytokines suggests that the sharing of the cytokine receptors might occur, because the cytokines are not expressed in same sites and thus would not limit the signaling of each other. Regulating the expression of just one receptor chain on the cell surface (IL-7Rα), will affect both. However, there are likely further lessons to be learned from TSLP and its functional receptors. Recently, neutrophils in mice were found to respond to TSLP (55), whereas at least in humans, neutrophils do not likely express IL-7Rα (56). It was recently also shown that dynamic IL-7Rα expression on DCs was required for IL-7 and TSLP responses (57), so one possibility might be that IL-7Rα is under very stringent regulation and is only upregulated in various cell types under very specific conditions.

## CONCLUDING REMARKS

Taken together, the organization and binding events of type I and type II IL-4 receptors have been reviewed here. The efficiency by which a functional IL-4/IL-13 receptor is formed appears to be a sum of three parameters. First, the binding efficiency of a cytokine to the cytokine-binding receptor chain dictates the concentration of the cytokine required for the saturation of the cytokine-binding receptor chain. Second, the binding efficiency of the cytokine/binding chain to the second receptor chain dictates the driving force for the completion of the receptor complex. Third, the expression level of the second receptor chain determines the availability of the second chains, at least in free fluid. All of these three parameters influence the efficiency of IL-4/IL-13 signaling and thereby tune the signal of the immune response in allergic inflammation.

## AUTHOR CONTRIBUTIONS

IJ planned and wrote the MS.

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# Seventeen-Year Journey Working With a Master

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It had been a great honor for me to work with the late Dr. William E. Paul for 17 years in the Laboratory of Immunology (LI) from 1998 until his passing in 2015. He was such a master in the immunology field. Under his outstanding guidance, my research has been focusing on transcriptional regulation of T helper (Th) cell differentiation, especially, on the role of a master transcription factor GATA3 during Th2 cell differentiation. Just as enormous scientific contributions of Dr. Paul (we all call him Bill) to the immunology community are far beyond his serving as the Chief of the LI, GATA3 also plays important roles in different lymphocytes at various developmental stages besides its critical functions in Th2 cells. In this special review dedicated to the memory of Bill, I will summarize the research that I have carried out in Bill's lab working on GATA3 in the context of related studies by other groups in the field of T cell differentiation and innate lymphoid cell (ILC) development. These include the essential role of GATA3 in regulating Th2/ILC2 differentiation/development and their functions, the critical role of GATA3 during the development of T cells and innate lymphoid cells, and dynamic and quantitative expression of GATA3 in controlling lymphocyte homeostasis and functions.

**Keywords: GATA3 transcription factor, T helper cells, innate lymphoid cells, cytokines, T cell differentiation, T cell development**

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## PREFACE

I joined the lab of Dr. William E. Paul (Bill) in 1998 as a postdoctoral fellow soon after I got my Ph.D. degree from the Shanghai Institute of Biochemistry, Chinese Academy of Sciences. Before I arrived in the U.S., Bill and I had already exchanged several emails regarding my potential projects. As a scientist who discovered interleukin (IL)-4, Bill had always been interested in IL-4 signaling and the structure of IL-4 receptor (IL-4R). He initially suggested me to crystallize the intracellular domains of the IL-4Rα chain, but I was more interested in transcriptional regulation of gene expression in lymphocytes, an area no one in Bill's lab had explored in the past. Bill later asked several other postdocs, who joined his lab after me, to work on IL-4R structure demonstrating his amazing persistence in research and impressive flexibility in mentoring.

Since IL-4 is the critical cytokine for driving type 2 T helper (Th2) cell differentiation (1), my first project started with searching for IL-4-inducible transcription factor(s) during early Th2 cell differentiation using DNA microarray. At the same time, I was working on the cross-regulation of T cell receptor (TCR)- and IL-4-mediated signaling (2) together with Dr. Hua Huang, a senior postdoc in Bill's lab at that time, who is now a full professor at the National Jewish Health.

My first project ended up with identifying growth factor independent-1 (Gfi-1) as an IL-4-inducible transcription factor, which plays an important role in promoting selective growth of committed Th2 cells (3). Later, Gfi-1 was also reported to suppress Th1, Th17, and Treg cell differentiation and the expression of IL-7 receptor α chain (4–6). The reason why we focused on transcription factors

**TABLE 1** | GATA3 expression and functions in distinct lymphocytes and during T cell/innate lymphoid cell (ILC) development/differentiation.

| Cell type or process | GATA3 levels | GATA3 functions | Genes regulated by GATA3 | Reference |
|---|---|---|---|---|
| T helper (Th)2 | High | Essential for Th2 cell differentiation and Th2 cytokine production; suppressing the expression of Th1-related genes | Inducing *Il4, Il5, Il13, Areg, Ccr8, Il1rl1, Il7r, Cd69*; suppressing *Ifng, Tbx21, Ccl5, Havcr2* | (7–9, 40, 41, 43, 44, 47, 50, 54, 85) |
| ILC2 | Very high | Essential for ILC2 development and type 2 cytokine production | *Il5, Il13, Areg, Ccr8, Il1rl1, Il7r, Il2ra, Il17rb* | (52–57) |
| Early T cell development | Intermediate to high | Essential for early T cell development | *Tcr*, *Cd3*??? | (59–61) |
| CD4 T cell development | Intermediate to high | Essential for CD4 but not CD8 T cell development | *Il7r, Cd3, Th-POK* | (47, 59, 62–64) |
| PLZF$^+$ ILC progenitor | High | Essential for the generation of PLZF/PD-1-expressing ILC progenitors | *Il7r, Id2*??? | (54, 65) |
| LTi progenitor | Low to intermediate | Required for LTi homeostasis and functions but not for development | *Il7r, Lta*??? | (54, 65) |
| Regulatory T cell | Low to high | Defining Treg subsets, modulating Treg functions and stability | *Il7r, Foxp3, Il2ra, Il1rl1, Ccr8* | (47, 66, 68–70) |
| Natural killer (NK)T cell | Low to high | Defining NKT cell subsets and maintaining homeostasis | *Il7r, Il4* | (74, 75) |
| CD8 T cell | Low | Homeostasis and memory generation | *Il7r, Myc* | (76) |
| Th1, Th17, and Tfh cell | Very low to low | Unknown | *Il7r, Ifng, Il4*??? | N.A. |
| ILC3 | Intermediate | Essential for the development of NKp46$^+$ ILC3s and modulating ILC3 function | Inducing *Il7r, Il22;* suppressing *Rorc* | (58, 65) |
| ILC1 | Intermediate | Homeostasis | *Il7r*??? | (26) |
| NK cell | Low | Maturation | *Ifng* | (72, 73) |

*??? refers to likely but not yet confirmed.*

that are induced by IL-4 at *early* stages of Th2 cell differentiation is mainly because, in 1997, Drs. Richard Flavell and Anuradha Ray's groups had already independently reported that GATA3 is necessary and sufficient for the expression of Th2 cytokines (7, 8).

In our initial report, the effect of Gfi-1 on Th2 cell proliferation was demonstrated by retroviral co-expression of Gfi-1 and GATA3 (3). To further assess whether Gfi-1 indeed plays an important role during Th2 responses under physiological conditions, with the help of Dr. Hua Gu who was a new Principle Investigator in the LI at that time, I started to generate Gfi-1 conditional knockout mice (4). At that time, GATA3 conditional knockout mice were not available either. While I was making Gfi-1 floxed mice, Bill gave me a very important suggestion—why don't you also prepare GATA3 conditional knockout mice at the same time (9). He said "I believe Gfi-1 is an interesting molecule to further work on, however, GATA3 is probably more important than Gfi-1 for Th2 cells." Indeed, throughout the 17 years period that I worked with Bill, first on T helper (Th) cell differentiation as a postdoctoral fellow and then on innate lymphoid cell (ILC) development as an independent investigator, I published 15 papers with their titles containing GATA3, but only 5 for Gfi-1. This visionary advice from Bill—always focusing on the most important things—has had a great impact on my research career.

## INTRODUCTION

CD4 Th cells orchestrate adaptive immune responses by producing effector cytokines. In order to effectively exert their protective functions during infections, distinct Th subsets are developed to deal with a variety of pathogens (10–12). There are three major Th cell subsets: type 1 T helper (Th1) cells that mainly produce IFN-γ, Th2 cells that produce IL-4, IL-5, and IL-13, and Th17 cells that produce IL-17a and IL-17f (13, 14). Th1 cells are important for immune responses to intracellular bacteria and viruses; Th2 cells are mainly responsible for immunity against helminth infections; whereas Th17 cells are essential for dealing with infections with extracellular bacteria and fungi. Besides their critical roles in mediating protective immunity, Th subsets are also capable of inducing many types of inflammatory responses. While Th2 cells are known to be involved in allergic diseases, Th1 and Th17 cells may cause autoimmunity (12, 15). All the Th effector cells are developed from naïve CD4 T cells when they encounter an antigen/MHCII complex that can be recognized by their antigen-specific TCR. Some naïve CD4 T cells may differentiate into regulatory T cells (Tregs) and they are regarded as peripheral induced Tregs (pTregs); together with thymic-derived Tregs, pTregs regulate the magnitude and duration of a particular immune response in addition to their essential role in maintaining immune tolerance (16–20).

In recent years, a group of non-B non-T lymphocyte-like cells that are capable of producing Th effector cytokines have drawn much attention in the field. These cells are now designated as innate lymphoid cells (ILCs) (21–24). Just like Th cells, there are three major ILC subsets: group 1 ILCs (ILC1s) that mainly produce IFN-γ, ILC2s that produce IL-5 and IL-13, and ILC3s that mainly produce IL-22. Since ILC subsets can produce cytokines known to be effector cytokines of Th cells, ILC and Th subsets of the same group are involved in related type of immune responses in a collaborative manner (25–30). For example, just as Th2 cells, ILC2s are not only involved in immune responses against helminth infections, but also induce allergic inflammation (29, 31–38). Therefore, similar to Th cells serving as professional cytokine-producing cells, ILCs are considered as the innate counterparts of Th cells.

The differentiation of Th1, Th2, and Th17 cells is mainly controlled by cytokine environment during their activation, which

induces the expression of lineage-defining transcription factors: T-bet for Th1; GATA3 for Th2; and RORγt for Th17 cells (39). These master regulators are not only essential for the differentiation and functions of Th subsets, but also they are utilized by ILC subsets for their development and functions: T-bet for ILC1s; GATA3 for ILC2s; and RORγt for ILC3s. While T-bet and RORγt are selectively expressed by Th1/ILC1 and Th17/ILC3 subsets, respectively, GATA3 is actually expressed by all the Th and ILC subsets although its expression in Th2 cells and ILC2s is the highest. Furthermore, GATA3 is dynamically expressed during T cell and ILC development. In this mini-review, I will discuss multiple important functions of this master transcription factor in a variety of lymphocytes at different developmental stages (**Table 1**).

## CRITICAL ROLE OF GATA3 IN Th2 DIFFERENTIATION AND FUNCTIONS

As mentioned earlier, Drs. Flavell and Ray's groups independently reported that GATA3 is a key transcription factor for inducing Th2 cytokine expression back in 1997 (7, 8). Soon after, Dr. Ken Murphy's group further showed that enforced expression of retroviral GATA3 induces endogenous GATA3 expression even in cells that were cultured under Th1 polarization conditions (40, 41). However, because GATA3-deficient CD4 T cells were not available at that time, direct evidence to support the essential role of GATA3 during Th2 differentiation particularly *in vivo* was still lacking. Nevertheless, these exciting reports inspired Bill and me to prepare a conditional knockout allele of *Gata3* by the Cre-loxP system (42). By using *Gata3* conditional knockout mice, both Dr. I-Cheng Ho's group and ours confirmed that GATA3 indeed is the master regulator of Th2 cells (9, 43). In the absence of GATA3, the production of Th2 cytokines is severely impaired, at the same time, IFN-γ production is induced even when the cells are cultured under Th2 conditions (44).

Interleukin-4-mediated STAT6 activation is sufficient to induce GATA3 expression during Th2 cell differentiation (45). Low dose of TCR stimulation can also upregulate GATA3 expression in the absence of IL-4 signaling (46). Indeed, Th2 differentiation may occur *in vivo* in an IL-4-STAT6-independent manner (15). On the other hand, although GATA3 can autoregulate its own expression, GATA3 is not required to induce itself in the presence of IL-4 signaling (47). Nevertheless, IL-4-dependent as well as IL-4-independent Th2 cell differentiation depends on GATA3 both *in vitro* and *in vivo* (9).

Genome-wide analyses of GATA3 binding through ChIP-Seq (chromatin immune-precipitation followed by high throughput sequencing) show that GATA3 binds to the Th2 cytokine locus *Il4/Il13* at multiple sites including sites in the *Il4* intron 2, the *Il13* promoter, and the locus control region within the *Rad50* gene (47). GATA3 also binds to the promoter of the *Il5* genes (48, 49). A major mechanism for GATA3 to induce IL-4 expression is through chromatin remodeling at the *Il4/Il13/Rad50* locus. In mature Th2 cells in which GATA3-mediated epigenetic modifications within the Th2 cytokine locus have already occurred, GATA3 is no longer needed for IL-4 production. However, since the activity of the *Il5* and *Il13* promoters always depends on GATA3, GATA3 deletion at any time completely abolishes IL-5 and IL-13 expression (9). Many other Th2-specific genes as well as long intergenic non-coding RNAs are also directly regulated by GATA3 (50). For example, T1/ST2, the IL-33 receptor encoded by the *Il1rl1* gene, is highly expressed in the most mature Th2 cells and GATA3 binds to the *Il1rl1* gene (47, 50).

## CRITICAL ROLE OF GATA3 IN ILC2 DEVELOPMENT AND FUNCTION

When I started my own research group, it had been known that there are a group of non-T non-B innate-like lymphocytes capable of producing type 2 cytokines and that type 2 cytokines produced by CD4 T cells are not essential for host defense (29, 33). Thus, I was very interested in what these cells were and how they developed. We hypothesized that GATA3, the critical factor for type 2 immune responses, may also be functionally important for the generation of type 2 cytokine-producing innate-like lymphocytes. Thus, we started to generate mice with GATA3 deficiency in the hematopoietic system and mice allowing inducible GATA3 deletion.

These innate-like cells are now known as type 2 innate lymphoid cells (ILC2s). Indeed, ILC2s express very high levels of GATA3 and they are highly enriched in the lung, skin, gut, and adipose tissues (21, 31, 35). Strikingly, ILC2s and Th2 cells generated during helminth infection are identical in their transcriptomes (51). Just as its critical function for Th2 cell differentiation, GATA3 is presumably also important for ILC2 development. However, due to its essential role during ILC development in the progenitor stage, which I will discuss later, definitive evidence showing the importance of GATA3 expression for ILC2 development is still lacking. Nevertheless, even in mature ILC2s, deletion of GATA3 results in loss of ILC2 functions (i.e., diminished IL-5 and IL-13 production) and reduced survival of ILC2s (52–57). Genome-wide analysis comparing transcriptomes between wild type ILC2s and GATA3-deficient "ILC2s" indicates that several important genes involved in type 2 immune responses, such as *Il5*, *Il13*, *Il1rl1*, and *Ccr8*, etc., are regulated by GATA3 (54). These genes are also regulated by GATA3 in mature Th2 cells, which may explain similar functionalities between ILC2s and Th2 cells.

GATA3 also directly binds to the *Il4/Il13* loci in ILC2s; the pattern of GATA3 binding to the Th2 cytokine locus in ILC2s is very similar to that in Th2 cells (47, 58). It has been reported that GATA3 regulates chromatin remodeling at several Th2-specific gene loci in Th2 cells (47), however, whether GATA3 play a similar role in epigenetic modifications in ILC2s is unknown. GATA3 also regulated the expression of the IL-33 receptor subunit T1/ST2 and IL-25R in ILC2s (47, 54, 58). Therefore, because of the downregulation of IL-33R and IL-25R expression in GATA3-deficient "ILC2s," these cells fail to respond to either IL-33 or IL-25. GATA3-deficient "ILC2s" also express lower levels of CD25 and IL-7R. Thus, there is a general defect of GATA3-deficient ILC2s in response to multiple cytokines.

## CRITICAL ROLE OF GATA3 IN T CELL AND ILC DEVELOPMENT

Besides its essential function in Th2 cells and ILC2s, GATA3 is also critical for T cell and ILC development at multiple stages (59, 60). GATA3 is important for the generation of T cell progenitors (59, 61). GATA3 is also required for CD4 but not for CD8 T cell development (47, 59, 62–64). Similarly, GATA3 is critical for the development of T helper-like ILCs that express IL-7Rα, but not of NK cells (54). In fact, high levels of GATA3 expression are required for the generation of PLZF/PD-1-expressing non-LTi progenitors but low levels of GATA3 expression are necessary for the function of LTi cells (65). Therefore, helper-like ILCs are considered as the innate counterpart of CD4 Th cells, whereas NK cells resemble innate CD8-like cells, and GATA3 is a master regulator for the development of both innate (ILC) and adaptive (Th) lymphocytes.

## CRITICAL FUNCTIONS OF GATA3 IN Tregs

GATA3 is also expressed by Tregs, and under certain circumstances, GATA3 expression may reach high levels, especially when cells receive IL-4 and/or TCR stimulation (66). The expression of some "Th2-related" genes, including *Il1rl1* and *Ccr8,* in Tregs depends on GATA3 (47). GATA3 binds to the *Foxp3* locus at the CNS2 region (67) and such binding may be important for maintaining optimal Foxp3 expression in Tregs (66, 68). Deletion of GATA3 specifically in Treg cells results in uncontrolled systemic Th2 responses in one study (68), however, other studies reported that these GATA3 conditional knockout mice were grossly normal although the GATA3-deficient Tregs showed some abnormal phenotype (66, 69, 70). Interestingly, GATA3 is dynamically expressed by Treg cells (70). Because persistent expression of GATA3 in Tregs at high levels may convert Tregs into Th2 cells (71), dynamic expression of GATA3 may be critical for maintaining Treg phenotype. Together with T-bet, GATA3 also suppresses RORγt expression in Tregs. Therefore, balanced expression of T-bet, GATA3, and RORγt in Foxp3-expressing is critical for Treg-mediated immune regulation (70).

## IMPORTANT FUNCTIONS OF GATA3 IN OTHER LYMPHOCYTES

GATA3 is expressed by ILC3s at intermediate levels (58). Interestingly, intermediate levels of GATA3 expression are required for regulating the balance between T-bet and RORγt, and thus the development of NKp46$^+$ ILC3s (58). GATA3 also regulates IL-22 expression in ILC3s (58). Whether GATA3 regulates the balance between T-bet and RORγt and/or IL-22 production in Th cells requires further investigation. GATA3 is also expression by ILC1s at intermediate levels and GATA3 is required for maintaining ILC1 homeostasis (26, 58). GATA3 is also expressed by NK cells but at low levels. Although GATA3 is not required for the development of conventional NK cells, it affects their maturation and cytokine production (54, 72, 73). GATA3 also affects NKT cell development and functions (74, 75) as well as CD8 T cell homeostasis partly through regulating IL-7Rα expression (76). Furthermore, GATA3 expression is found at low levels in Th1 and Th17 cells; however, its functions in these cells require further investigation.

## RELATIONSHIP BETWEEN GATA3 AND OTHER IMPORTANT TRANSCRIPTION FACTORS

During Th2 differentiation, GATA3 can be upregulated by IL-4/STAT6 and/or TCR-mediated signaling (15). However, ILC2 development does not require IL-4/STAT6 signaling. It is possible that Notch signaling plays an important role in GATA3 induction in ILCs. Consistent with this notion, TCF7, a transcription factor induced by Notch signaling, can upregulate GATA3 expression in ILC progenitors (56, 77). What induces/maintains high GATA3 expression in ILC2s is not known.

Although GATA3 plays an essential role in the development and functions of ILC and Th cell subsets, many other transcription factors, including Id2, TCF7, Tox, and Th-POK may form a network with GATA3 in determining cell lineage fates (26, 63, 77, 78). Just as GATA3, Bcl11b is important for the development of T cells and ILC2s (79–84). We have recently reported that GATA3 and Bcl11b form a complex and they co-localized in many enhancer regions within the Th1- and Th2-related genes (85). Interestingly, the GATA3/Bcl11b complex not only suppresses the expression of many Th1-related genes, but it also controls the magnitude of Th2 responses. GATA3 and Bcl11b may have common targets in ILC2s, which requires further investigation.

Several other transcription factors can also interact with GATA3. T-bet interacts with GATA3 and suppresses its function (86, 87). Consequently, T-bet and GATA3 co-bind to many Th1- or Th2-related genes (88–90). T-bet overexpression suppresses GATA3 expression at the transcription level (87). Endogenous expression of T-bet may also inhibit a GATA3-mediated "default" Th2 program during Th1 cell differentiation (90). Interestingly, T-bet is detected in GATA3-expressing cells during helminth infection to limit Th2 responses (91). On the other hand, GATA3 may silence the *Tbx21* gene during Th2 cell differentiation (47). GATA3 may also inhibit Th1 differentiation by suppressing the expression of STAT4 expression as well as Runx3-mediated induction of IFN-γ expression (44, 92), and GATA3 can bind to Runx3 at the protein level.

## CONCLUSION AND FUTURE DIRECTIONS

Bill was the master of the Laboratory of Immunology at the NIAID, NIH. I had learned tremendously from him through weekly one-on-one meetings throughout the 17-year period working with him. In the earlier era, Bill had also trained many world renowned immunologists, including Drs. Charles Janeway, Mark Davis, Laurie Glimcher, and Ronald Schwartz. Not only Bill had trained many incredible scientists in his lab, but also he had a great impact on our immunology community at the NIH and around the world. Thus, Bill is a true master of immunology. Without him, the NIH immunology interest group has been

suffering from a "knockout" phenotype in the past 2 years. We sincerely hope that a master(s) with his/her knowledge and ability equivalent to Bill's will inspire our community again in the near future.

The master regulator for Th2 cells is GATA3. Just like Bill who contributed to the immunology field in many aspects, GATA3 also plays an essential role during early T cell development, CD4 T cell development as well as ILC development. In mature lymphocytes, ILC2s followed by Th2 cells express the highest levels of GATA3, which is consistent with its critical function in maintaining the functionalities of these type 2 lymphocytes. In other lymphocytes, including Tregs, NKT cells, CD8 T cells, ILC1s, ILC3s, NK cells, and possibly Th1 and Th17 cells, GATA3 may also regulate their homeostasis and functions (**Table 1**). GATA3-mediated IL-7Rα induction may be a common mechanism through which GATA3 regulates lymphocyte homeostasis; however, this may not fully explain the multifunctions of GATA3 during T cell and ILC development (58, 76).

Because GATA3 is expressed by all T cell and ILC subsets, and its expression varies from cell type to cell type and from stage-to-stage, the functions of GATA3 in different lymphocytes at various developmental and activation stages may be controlled by its expression levels and its interacting partners. Quantitative expression of GATA3 may result in a qualitative effect. To study GATA3 dose effect, a model with a titratable GATA3 expression may be needed to separate the differential roles of GATA3 expressed at high or low levels during the development of T cells and ILCs. Distinct complexes containing GATA3 in different cell types may offer cell-type-specific gene regulation. Thus, identifying GATA3-interacting proteins in different lymphocytes will help us understand the mechanisms of GATA3-mediated gene regulation, which will guide us to obtain deeper insights into the biology of the immune responses in allergic, infectious, autoimmune, and other inflammatory diseases.

We have recently generated a new GATA3 reporter mouse strain through the CRISPR/Cas9 technology by inserting a ZsGreen-T2A cassette into the *Gata3* conditional allele flanked by two LoxP sites. This novel reporter works beautifully: variable GATA3 expression ranging for several logs in GFP intensity is observed in distinct lymphocytes at different developmental stages. We are using this mouse strain in combination with different Cre transgenic lines to study the function of this master regulator in a variety of lymphocytes particularly *in vivo*. We will be happy to share this valuable mouse strain with other labs that are interested in using it, even before its publication, as Bill had taught us the right way to promote science. Although Bill is no longer with us, and I cannot discuss our new exciting results with him anymore, my fascination in studying "master regulators" inspired by Bill will continue endlessly and I believe that is what Bill had hoped for the new generation(s) of immunologists.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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# TCR Signaling Abnormalities in Human Th2-Associated Atopic Disease

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Stimulation of naïve CD4 T cells with weak T cell receptor agonists even in the absence of T helper-skewing cytokines can result in IL-4 production which can drive a Th2 response. Evidence for the *in vivo* consequences of such a phenomenon can be found in a number of mouse models and, importantly, a series of monogenic human diseases associated with significant atopy which are caused by mutations in the T cell receptor signaling cascade. Such diseases can help understand how Th2 responses evolve in humans, and potentially provide insight into therapeutic interventions.

**Keywords: T-cell receptor repertoire, signaling pathways, primary immunodeficiencies, atopic disease, monogenic syndromes**

## INTRODUCTION

Within the vast legacy of Bill Paul's career, one theme that emerged was the search for a source of IL-4 that would meaningfully provide differentiating naïve CD4 T cells sufficient signal to develop into memory Th2 cells during an immune response that required such a program. Mast cell (1), basophil (2–4), and NK T (5) IL-4 production were observed, but whether they are the key initiators of most Th2 responses continues to be a matter of debate. It was therefore in the course of that search that attention was turned to IL-4 production by the naïve T cell itself (6, 7). Kim Bottomly, herself a trainee of Bill's, had observed that *in vitro* priming of naïve T cells by relatively weak, but not strong, agonist peptides, in the absence of other priming cytokines, could lead to a Th2 response (8–15). Bill's lab later showed that "strong" agonist peptides themselves could prime such a response, when provided at a sufficiently low dose (16), and that in lymphopenic states or when TCR of high affinity for a given peptide are removed, stimulated naïve cells will differentiate into Th2 cells (17, 18). The mechanisms for these observations continue to be unraveled, but include the notion that responses to IL-2 become blunted at higher dose of peptide, preventing the necessary STAT5b activation and nuclear translocation for transcription of key Th2 lineage transcription factor as well as poor ERK activation, as MEK inhibition could recapitulate the Th2 bias even in the presence of high dose strong TCR agonism (9, 16).

*In vivo*, indeed TCR/MHC interactions may even predominate over exogenous adjuvant activity in determining Th1/Th2 balance (19), although it may not always be *via* IL-4 production itself (20). One potential teleologic reason for the phenomenon could be that parasitic products which could evade immune responses by downregulating TCR-MHC interactions [such as the omega-1 component of schistosome egg antigen which can prime Th2 responses, potentially by weakening TCR/MHC interactions (21, 22)] resulted in the evolution of anti-parasitic cytokine profiles which are derived from differentiation under low-affinity conditions. Whatever the cause, and whether IL-4 itself is the key driver of Th2 differentiation *in vivo* is a matter of debate, the success of IL-4 receptor blocking antibodies in treating human atopic disease has been impressive, strongly suggesting this pathway is critical for the pathogenesis of human atopic disease (23).

Another set of observations have further buttressed the notion that altered TCR signaling could lead to Th2 phenotypes. A series of mouse lines derived spontaneously or *via* random mutagenesis

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with missense mutations in key TCR signaling molecules were observed to develop Th2-related pathology spontaneously. These included LAT, ZAP70 (in several independent mutant lines), and CARMA1 (24–28). Null mutations in most of these molecules lead to impairment of effector function which precludes most Th differentiation altogether, and as such it is the hyopomorphic loss-of-function mutations which lead to the phenotype.

Of course, a major consequence of this basic observation could be that certain human disease could also be driven by this phenomenon and would most likely include an atopic phenotype. With the exponential growth of patients undergoing next-generation sequencing, multiple newly described immune disorders which include atopic disease have been identified, some of which may well be due to impaired TCR signaling. This review therefore provides a series of examples of human monogenic disorders associated with atopy which may be caused by imbalances in TCR signaling which fail to prevent Th2 responses.

## OMENN SYNDROME (OS)

Before directly addressing the propensity for mutations to intrinsically bias a T cell toward Th2 differentiation, it is critical to distinguish one congenital atopic phenotype, namely, that seen in OS (29). Mutations that are known to lead to massive curtailment of T cell function and/or number—both intrinsic to signaling and extrinsic to it—can nonetheless permit "leaky" peripheral T cell populations which can progress to CD4 lymphoproliferation, organomegaly, and Th2-like disease associated with marked IgE elevation, erythroderma, and eosinophilia. Why OS is associated with the Th2 phenotype is not clear, but hypotheses have included a failure of central tolerance due to abnormal thymic development which hinders both AIRE-induced negative selection and the generation of a normal repertoire of FOXP3+ regulatory T cells (Tregs) (30–32). The lymphopenic state also may lead to the absence of sufficient high-affinity competition for antigen which would then permit low-affinity cells to be stimulated and proliferate, leading to the Th2 phenotype (17, 18).

## MUTATIONS IN GENES ENCODING CLASSICAL TCR SIGNALING PROTEINS

Similar to the mouse, human mutations in ZAP70 can lead to varied phenotypes from SCID, to autoimmunity, to highly atopic phenotypes (33–37). In the case of one of the reported atopic phenotype in humans, it is not clear whether it was caused by intrinsic Th2 bias similar to the mouse model, or due to the limited repertoire associated with OS (35).

Stronger evidence for the link between TCR intrinsic signaling defects and atopy in human disease can be found in hypomorphic mutations of two members of the CBM complex, such as MALT1 (38) and CARMA1 (39, 40). The CBM complex, which includes MALT1, CARD11, and BCL10, is required for normal NFkB activation after TCR ligation, as well as mTORC1 activation (41, 42). Complete loss-of-function mutations of any of the three CBM complex members lead to a SCID-like illness (43–47), but recently, hypomorphic MALT1 mutations were described in a patient with recurrent infection, marked IgE elevation, and severe eczema (38). Even more recently, dominant-negative mutations leaving residual, hypomorphic CARD11 activity were identified in a cohort of patients with severe atopic disease with, and in some cases, without, comorbid infection. The finding is of particular interest since, in addition to the possibility that severe atopy without comorbidity could be explained by a single-gene mutation, CARD11 has been identified in GWAS studies of common atopic dermatitis (48).

While numerous patients with defects in nearly every NFkB subunit have been identified, atopy has not been reported to be associated with any of them. The lack of atopy argues that defects in another pathway in which CARD11 is involved might explain the allergic disease these patients have. Recent evidence suggests that CARD11 may also participate in mTORC1 activation (42) by recruiting, upregulating, and/or activating of the glutamine transporter ASCT2, which in turn leads to increased intracellular glutamine needed for mTORC1 activation. ASCT2−/− mice have a Th2 phenotype (49), potentially due to inadequate glutamine transport, which may be required for normal Th1 differentiation and the prevention of excessive Th2 differentiation (50, 51). The CARD11DN patients have evidence of impaired mTORC1 activation and reduced Th1 cytokine production, rescuable by exogenous glutamine (39), raising the possibility that glutamine supplementation could be of clinical benefit in these patients. Of note, glutamine supplementation of premature infants is associated with protection from the development of atopic dermatitis (52, 53).

## MUTATIONS IN GENES ENCODING ACTIN CYTOSKELETON PROTEINS

Following TCR ligation, Wiskott–Aldrich syndrome protein (WASP) dissociates from its stabilizing partner WASP-interacting protein (WIP) and binds actin-related protein (ARP) 2/3 (54) to begin the actin assembly cascade.

Loss of WASP leads to Wiskott–Aldrich syndrome, which is characterized by severe atopic dermatitis, increased gut sensitization and clinical food allergy, thrombocytopenia, and combined immunodeficiency (55, 56). A similar phenotype occurs with loss of WASP-interacting protein family member 1 (*WIPF1*) encoding WIP (57) as well as an ARP2/3 subunit, actin-related protein 2/3 complex subunit 1B (*ARPC1B*) (58–60).

WASP-interacting protein also appears to associate with dedicator of cytokinesis 8 (DOCK8) a guanine nucleotide exchange factor whose activity is critical for normal WASP function (61). Loss of function in DOCK8 leads to significant elevations in IgE, combined immunodeficiency, and other many clinical features in common with WAS, including severe atopic dermatitis and food allergy, and even autoimmunity (62, 63). Thrombocytopenia is not seen in DOCK8 deficiency, while severe viral skin infections and anaphylaxis are not as common in WAS, potentially due to differences in redundancy, function, and tissue expression (56, 64, 65).

Once again, we know less about why Th2 phenotypes emerge from these actin cytoskeleton-related mutations. DOCK8 patient lymphocytes have a T cell-intrinsic bias toward Th2, and away

from Th1 differentiation (66), and WASP transcriptional activity appears to be critical for Th1 differentiation (67, 68). Another possible mechanism suggests these proteins have critical roles in Treg function, potentially *via* IL-2 activity, the impairment of which therefore would lead to immune dysregulation of all types, including Th2 (56, 69–73).

On this point, it is important to note that Treg failure is always a consideration when trying to understand how impaired TCR signaling could lead to Th2 phenotypes, since an ideal TCR signal is necessary for normal Treg development, differentiation, and function (74). While CARD11DN patient Tregs appeared quantitatively and qualitatively normal, the mouse model suggested otherwise (26). It is further noteworthy that while the mechanism of weak TCR signal failing to curtail STAT5b activity has not yet been studied in the human TCR signaling defects, gain-of-function missense mutations in STAT5b, and JAK1—which activates STAT5b—are associated with syndromes characterized by profound early onset dermatitis and eosinophilia (75, 76). That said, while STAT5bGOF mutations lead to a Th2 phenotype, so too can STAT5bLOF mutations, which are associated with severe Treg impairment (77). While in humans it is difficult to tease apart the relative contributions of effector T cell intrinsic predisposition toward Th2 responses and responsiveness to extrinsic regulation from the number and function of Tregs themselves, it is still important to study both in the context of human diseases of impaired TCR signaling.

## CONCLUSION

A great deal remains unknown or unproven with respect to the direct role for TCR signaling defects and/or weak TCR signaling in human allergic disease. The limitations which exist when studying human T helper differentiation make it harded to directly demonstrate causality. However, the preponderance of evidence coupling mouse and human *in vitro* studies with *ex vivo* human studies suggests disruption of a number of TCR signaling pathways could well lead to a Th2 phenotype which in turn drives an organismal atopic disease. Apart from the mechanistic insight this provides, how such knowledge could be translated into positive therapeutic manipulation remains a question. Balancing the therapeutic manipulation with risk and cost is of course key. While indeed targeting Th2 cytokines has been quite successful in the clinic, the use of such medications is still in its early phases, and they are extremely expensive. Of course, depending on the severity of disease, bone marrow transplant can be an option, and in theory so could gene therapy and/or gene editing. Other interventions meant to strengthen TCR signaling always run the risk of leaning toward aberrant autoreactivity as well. The ultimate consequences of these balances and their perturbation will be gleaned from continued mechanistic research into the precise mechanisms by which the Th2 phenotypic program emerges when TCR signaling is impaired.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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# Spontaneous T Cell Proliferation: A Physiologic Process to Create and Maintain Homeostatic Balance and Diversity of the Immune System

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Naive T lymphocytes undergo heterogeneous proliferative responses when introduced into lymphopenic hosts, referred to as "homeostatic proliferation" and "spontaneous proliferation." Spontaneous proliferation is a unique process through which the immune system generates memory phenotype cells with increasing T cell receptors repertoire complexity. Here, the mechanisms that initiate and control spontaneous proliferation are discussed.

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The immune system is constantly exposed to foreign antigens derived from microbes that are either harmless or pathogenic and to self-antigens that are either normal or transformed. The fundamental goal of such immunological practices is to achieve two outcomes: to eliminate harmful pathogens and transformed cells while to preserve harmless commensal microbes and normal cells.

T cells are a trustworthy fighter that identifies and eliminates invading pathogens, yet a potential traitor capable of attacking self-tissues and causing autoimmunity. The immune system has thus evolved several lines of checkpoints that ensure safety and loyalty of developing lymphocytes. Thymocytes that express useless or harmful antigen receptors are eliminated by apoptotic cell death, while those expressing self-MHC restricted antigen receptors with moderate reactivity to self-antigens are allowed to mature. T cells that survive the selection processes and leave the thymus to express the T cell receptors (TCR) displaying a measurable, yet weak reactivity against self-antigens that is not strong enough to cause autoimmunity, yet sufficient enough to maintain both survivability and reactivity to the subsequent antigen encounter (1, 2). Those T cells selected by relatively stronger interaction toward self-antigens may further develop into T cells with regulatory functions (3–5). In the periphery, T cells constantly receive a "tonic" signal from interacting with $MHC^+$ antigen presenting cells (APCs), resulting in a partial phosphorylation of the ζ chain of the TCR complexes (2). This is critical to maintain proper reactivity and survival of T cells, although the impact on the latter remains controversial (2, 6). Since developing thymocytes are selected based on "self-reactivity," the immune system develops an additional measure to prevent unnecessary T cell activation in response to self-antigens in the periphery, including anergy and regulatory T cells. The failure in such regulatory mechanisms results in uncontrolled autoimmune inflammatory responses. Thus, keeping the regulation under control is a matter of utmost importance.

The number and composition of T cells in the periphery is tightly controlled at a relatively constant level throughout life, suggesting the existence of a homeostatic mechanism(s). Alterations in the homeostasis trigger series of compensatory mechanisms that reinstate homeostatic equilibrium. For example, viral infection causes clonal expansion of antigen-specific CD8 T cells, during which peripheral CD8 T cells massively expand and up to ~90% of the total CD8 T cells may become

antigen specific in case of lymphocytic choriomeningitis virus infection (7). Once the infection is cleared, homeostasis returns to the normal level as the majority of the expanded effector cells are eliminated, leaving newly generated virus-specific memory T cells. Lymphopenia incurred from normal physiologic processes that occur during neonatal period, from pathogenic conditions, such as viral infection, or from therapeutic interventions triggers T cell proliferation that restores the T cell deficiency (8–10). Therefore, T cell homeostasis is a key process that requires a precise balance between proliferation and apoptosis. A plethora of evidence indicates that dysregulation in T cell homeostasis can lead to inflammatory disorders, including autoimmune diseases, HIV-associated immune reconstitution inflammatory syndrome, Omenn syndrome, bare lymphocyte syndrome, and others (11–14).

## T CELL PROLIFERATION IN LYMPHOPENIC SETTINGS, A MODEL TO STUDY T CELL HOMEOSTASIS

For the past decades, examining T cell proliferation under lymphopenic settings has been the primary *in vivo* model system to investigate mechanisms controlling T cell homeostasis and immunopathology associated with homeostatic imbalance. Pioneering studies from the Chen and Bevan groups demonstrated that naive CD8 T cells transferred into irradiated or $Rag^{-/-}$ recipients undergo proliferative responses without "cognate antigen" stimulation and acquire a memory-like phenotype (15, 16). The proliferative potential of such responses was once estimated that one T cell has a potential to generate $10^{15}$ progenies during the process (17).

## SPONTANEOUS PROLIFERATION VS. HOMEOSTATIC PROLIFERATION

While earlier studies interchangeably utilized mild and severe lymphopenic models to investigate proliferative T cell responses inclusively called homeostatic proliferation (or lymphopenia-induced proliferation), subsequent studies uncovered that T cell proliferation within lymphopenic settings is highly heterogeneous. We reported that there are at least two mechanistically distinct proliferation modes referred to as spontaneous proliferation and homeostatic proliferation (18). Spontaneous proliferation is a robust proliferation found in "severe" lymphopenic hosts, including mice with mutation in genes involved in lymphocyte generation. Spontaneously proliferating cells divide more than a cell division per day even in the absence of homeostatic cytokines (18, 19). In case of CD4 T cells, the requirement for spontaneous proliferation is rather unique, because MHC II molecules expressed on $CD11c^+$ dendritic cells (DCs), but not on B cells are required for proliferation (20). The requirement for naive CD8 T cell spontaneous proliferation is less rigorous, and either MHC I or MHC II expressed on DCs or B cells are sufficient to induce proliferation (20). Additional important feature for spontaneous proliferation is that the proliferating cells turn into phenotypically different populations. They rapidly differentiate into memory phenotype cells, acquiring memory/effector cell markers and an ability to produce inflammatory cytokines upon stimulation (18). Unlike T cells activated by cognate antigen, however, spontaneously proliferating T cells do not express early activation markers (CD69 and CD25), although CD44 upregulation and CD62L downregulation still occurs, allowing them to preferentially migrate into non-lymphoid tissues as antigen-stimulated effector/memory T cells do. Homeostatic proliferation is a slow response that occurs within "mild" lymphopenic conditions following sublethal irradiation or T cell ablation in the presence of functionally intact thymus (18, 21). Homeostatically proliferating CD4 T cells undergo a cell division every 3–4 days, although CD8 T cell proliferation is considerably faster than that of CD4 T cells (18). TCR interaction with MHC:peptide complexes is instrumental for the responses as blocking the interaction inhibit proliferation (22, 23). However, TCR engagement alone is not sufficient for proliferation. Treatment with neutralizing antibodies against homeostatic cytokine, namely IL-7, significantly inhibits homeostatic proliferation of T cells (18). Therefore, signals generated from the TCR and the cytokine receptors must be incorporated to trigger proliferation. The nature of antigens involved in homeostatic proliferation remains unclear. However, it is likely low affinity self-antigens because homeostatic proliferation is not impaired in germ-free lymphopenic recipients (19).

## QUANTITATIVE AND QUALITATIVE SIGNALING MODELS

To account for the distinct nature and underlying mechanisms underlying homeostatic and spontaneous proliferation we propose the quantitative and qualitative signaling models (**Figure 1A**). The quantitative signaling model for homeostatic proliferation postulates that the relative amount of available resources determines the mode of T cell proliferation. The level of serum IL-7 is found significantly higher in lymphopenic hosts (24, 25). In fact, IL-7 production by stromal cells appears to be controlled as a part of homeostatic mechanism (24), through which peripheral T cell survival, proliferation, and apoptosis are balanced. In addition, the relative abundance of lymphocytes in the periphery may further determine the competition. In $Rag^{-/-}$ recipients, a low competition (i.e., more availability) for IL-7 promotes cell survival by enhanced expression of anti-apoptotic factors and cell proliferation by degrading cell cycle inhibitor p27 (26). Homeostatic proliferation is a dominant response in these environments. However, the level of IL-7 available is likely lower in $TCR\beta^{-/-}$ or TCR transgenic mouse recipient due to competing endogenous B cells or transgenic T cells. Due to competition for IL-7, homeostatic proliferation is not typically observed in these recipients (18, 27). However, provision of exogenous IL-7 induces homeostatic proliferation in such conditions, supporting the importance of IL-7 during homeostatic proliferation. Moreover, the extent of proliferation is similar to that observed in $Rag^{-/-}$ or sublethally irradiated recipients and is proportional to the amount of given IL-7 (18). T cells transferred into lympho-replete wild type recipients remain undivided, and providing exogenous IL-7 is sufficient to trigger homeostatic proliferation of the transferred cells in lymphocyte-sufficient environments (18).

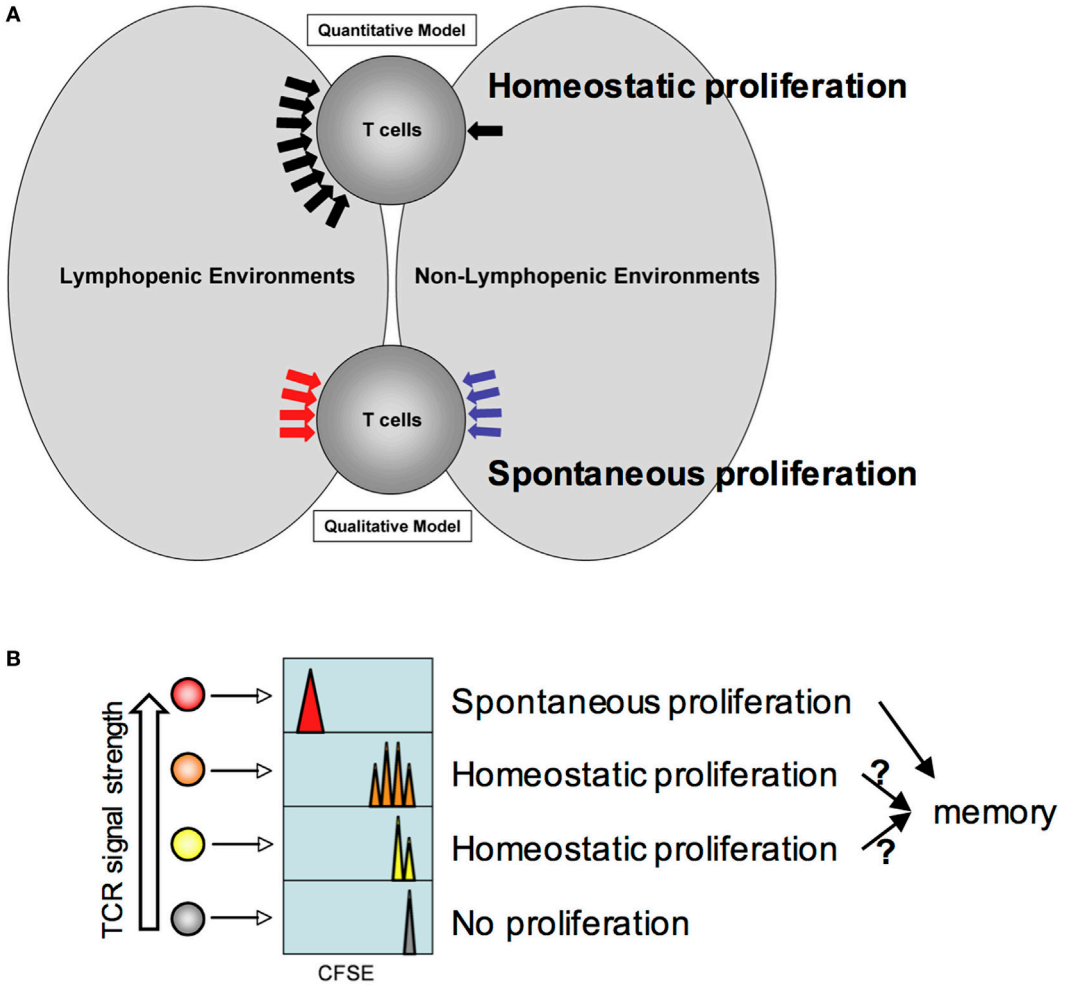


**FIGURE 1** | Model for homeostatic and spontaneous proliferation. **(A)** Quantitative and qualitative signaling model. The model depicts potential signaling mechanisms during homeostatic and spontaneous proliferation. Homeostatic proliferation is triggered by excessive soluble resources available under lymphopenic environments. By contrast, spontaneous proliferation is triggered by different types of signaling mechanism only available under lymphopenic conditions. **(B)** Relative T cell receptors signal strength against endogenous peptide:MHC complexes determines which T cells undergo homeostatic and spontaneous proliferation. The higher the strength is, the more likely the T cells would undergo spontaneous proliferation. If the strength is below threshold, cytokine availability controls homeostatic proliferation. T cells would remain undivided.

The qualitative signaling model for spontaneous proliferation postulates that the nature of signals that T cells receive is fundamentally different from those that T cells receive within lymphocyte-sufficient conditions (**Figure 1A**). IL-7 or other homeostatic cytokines play little or no role in this response. Instead, antigens originated from commensal microbes appear essential in inducing spontaneous proliferation as proliferation is considerably reduced in germ-free lymphopenic animals (19). However, it is worth noting that a measurable spontaneous proliferation is still observed in germ-free lymphopenic hosts, suggesting a contribution of food or self-antigens. Given the fast proliferation dynamics and full differentiation, it is likely that the signaling cascade may be analogous to that of cognate antigen-induced T cell activation.

The molecular basis underlying spontaneous and homeostatic proliferation has been tested using various gene knockout animals. Zamoyska and colleagues reported using the $p56^{lck}$-deficient system that sustained lck expression is required for the proliferation of CD4 and CD8 T cells (28). Lck deficiency in transferred T cells impairs homeostatic and possibly spontaneous proliferation of both CD4 and CD8 T cells (28). However, the study was carried out over 3–6 weeks post transfer. The study is thus not suitable to determine the exact requirement of lck during spontaneous proliferation. Shen et al. examined the importance of the LAT (linker for activation of T cells) during T cell proliferation in lymphopenia (29). They also measured $LAT^{-/-}$ T cell proliferation in T cell-deficient $LAT^{-/-}$ recipients, where T cell development is blocked. LAT deficiency greatly impairs T cell proliferation, suggesting that LAT expression may be necessary for spontaneous proliferation (29). Gascoigne and colleagues examined the role of different protein kinase C isoforms and found that PKCη, but not PKCθ plays a key role in regulating homeostatic proliferation

of CD8 T cells in sublethally irradiated recipients (30). Signaling pathways that occur during spontaneous proliferation remain to be investigated.

Does each T cell express equal potential to undergo homeostatic and/or spontaneous responses and are they stochastically selected? Or alternatively, are they predetermined for either but not for both responses? The strength of "tonic" signals may be a key factor determining which cells are selected to respond to homeostatic or endogenous cues. Peripheral mature T cells are a highly heterogeneous population such that each T cell clone expresses the antigen receptors with spectral affinity against self (and/or commensal) antigens. The heterogeneity (i.e., the strength of such interaction) can be reflected by the level of surface expression of CD5, a negative regulator of TCR signaling (31). An earlier study where $CD5^{hi}$ or $CD5^{lo}$ naïve T cells were purified and transferred into mild lymphopenic recipients showed more extensive homeostatic proliferation of $CD5^{hi}$ T cells in sublethally irradiated recipients (32). Therefore, T cells expressing higher affinity antigen receptors are likely to participate in spontaneous proliferation, although this hypothesis needs to be tested. The proliferating cells turn into memory phenotype cells and play a central role in regulating spontaneous proliferation of naive T cells (see below). On the other hand, T cells expressing low or moderate affinity receptors are expected to undergo homeostatic proliferation or remained undivided (**Figure 1B**). Interestingly, a correlation between slow cell division and CD44 upregulation was observed during homeostatic proliferation (18). These cells may eventually differentiate, if allowed, into memory phenotype cells analogous to those generated from spontaneous mechanism and participate in enhancing the memory cell repertoire complexity (see below). Alternatively, there may exist a threshold that allows proliferating cells to become functional memory cells (33). Marginal upregulation of CD44 expression in homeostatically proliferating cells may become transient and reversible as previously observed (15). $Foxp3^{+}$ regulatory T cells are thought to be selected from developing T cells recognizing self-antigens with a higher affinity (34–36). Indeed, Tregs or $CD44^{hi}$ memory phenotype CD4 T cells are known to undergo more extensive spontaneous proliferation even within lymphocyte-sufficient environments (37, 38).

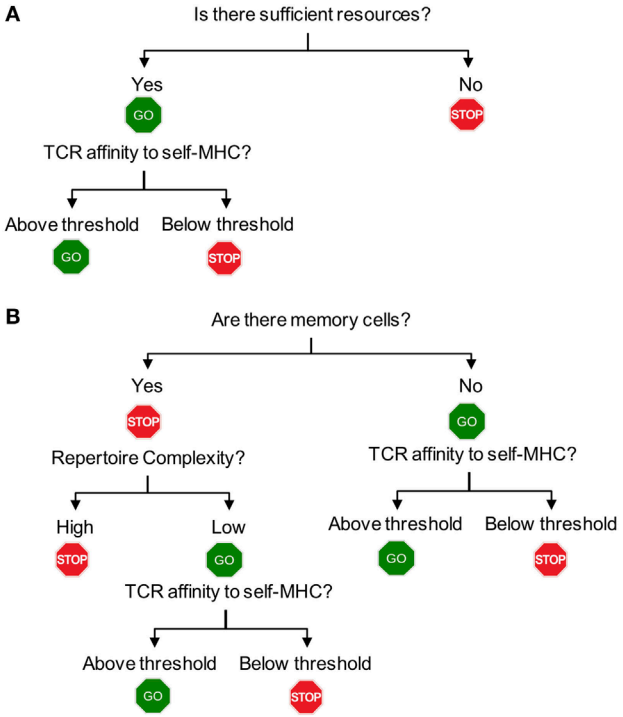


**FIGURE 2** | Flowchart for homeostatic and spontaneous proliferation. **(A)** Homeostatic proliferation. The first step for T cells to check is whether there is sufficient homeostatic factor available. Once the source is available, then the relative affinity of each T cells expresses against MHC-self-antigens (or exogenous antigen as well) will determine proliferation. **(B)** Spontaneous proliferation. Unlike homeostatic proliferation, the first step deciding spontaneous proliferation is whether there is a memory population in the periphery. The lack of any memory population triggers a full blown spontaneous proliferation during which T cells with higher affinity to MHC-self-antigens (and commensal antigens) are induced for proliferation. If there is a population of pre-existing memory cells, the complexity of T cell receptors (TCR) repertoire will then become the next step determining proliferation. Only incomplete repertoire complexity of memory cells will allow spontaneous proliferation to occur. The final decision will depend upon the TCR that each T cell expresses. Only if the TCR strength toward endogenous peptides (self or commensal) is above threshold, they will be allowed to undergo proliferation.

## PRINCIPLES FOR SPONTANEOUS PROLIFERATION

Examining spontaneous proliferation has uncovered several unique features that are distinct from those operated during homeostatic proliferation (**Figure 2**). *First, spontaneous proliferation is triggered by the lack of memory T cells not by the total T cell numbers*. The earlier notion accounting for T cell proliferation within lymphopenic environments was that T cells "sense" the existence of neighboring T cells. Homeostatic proliferation competing for soluble resources is properly explained by the notion. However, the finding that spontaneous proliferation is comparably induced when T cells are transferred "into" TCR transgenic recipients, where wild type level peripheral T cells with monoclonal TCR repertoire and primarily naive phenotype exist strongly suggests that the total number of peripheral T cells is not a factor for proliferation. In fact, the absence of memory T cells in this condition is the signal triggering proliferation. *Second, the clonality of peripheral T cells determines spontaneous proliferation*. However, it was further shown that the presence of memory cells itself cannot be a sole factor determining spontaneous proliferation (**Figure 2B**). TCR transgenic T cells undergo spontaneous proliferation when transferred to TCR transgenic hosts with a different clonotype, while remain undivided when transferred to the hosts with the same clonotype, demonstrating the importance of TCR clonality (39). One may also postulate that memory T cells may better compete for homeostatic resources, thereby efficiently inhibiting spontaneous proliferation. Then, it is predicted that TCR transgenic recipients with abundant memory cells (of the same specificity) would be sufficient to control the proliferation. Immunizing TCR transgenic mice with cognate antigens by which large proportions of peripheral T cells

become effector/memory cells is unable to inhibit spontaneous proliferation of polyclonal naïve T cells (40), suggesting that the clonality of memory T cells is crucial. *Third, the repertoire complexity of memory T cells controls spontaneous proliferation.* Varying numbers of T cells transferred into immunodeficient mice form memory phenotype cells with different repertoire complexity. Importantly, the total number of memory phenotype cells generated from different T cell inoculums' remain relatively similar, suggesting that there appears to be a homeostatic mechanism that maintains the size of memory T cells (40). When the second cohorts of naïve T cells are introduced into these recipients, the extent of spontaneous proliferation of newly transferred cells is directly determined by the repertoire diversity of the pre-existing memory cells (40). When pre-existing memory T cell repertoire complexity is low, the extent of new spontaneous proliferation from the second naïve T cells is greater. By contrast, pre-existing memory T cells with higher repertoire complexity efficiently limit spontaneous proliferation of new naïve T cells. Direct comparison of the TCR clonality between the pre-existing and newly formed memory phenotype cells demonstrates that their TCR clonality is mostly non-overlapping (40). Therefore, it is concluded that a "hole" in the memory T cell repertoire allows naive T cells that are capable of recognizing antigens occupying the hole to undergo spontaneous proliferation and to differentiate into memory cells (41). As the result, the "hole" would be filled up by these newly formed memory phenotype cells, increasing the overall repertoire complexity and becoming homeostatically "stable."

## REGULATION OF SPONTANEOUS PROLIFERATION

The fact that spontaneous proliferation is diminished in germ-free immunodeficient hosts strongly suggests that antigens derived from commensal organisms are the primary source supporting proliferation and differentiation. However, the very similar commensal antigens are also likely presented in a lympho-replete condition possibly in a tolerogenic fashion. *Why do commensal antigens fail to trigger spontaneous proliferation in this case?* We already discussed that the presence of memory phenotype cells with higher repertoire complexity controls the induction of spontaneous proliferation. *Then, how does it operate?*

Reconstituting immunodeficient mice with two cohorts of T cells at different time points gives us a system, where one could examine the underlying cellular mechanisms. Pre-existing memory phenotype CD4 T cells limit spontaneous proliferation of both naive CD4 and CD8 T cells (23). The hypothesis is that the memory T cells inhibit naive T cell proliferation *via* altering APC functions. By reconstituting mice with a mixture of TCRβ$^{-/-}$ bone marrow progenitors that express MHC II or not, we create a mouse model in which half of the bone marrow-derived APCs express MHC II (and the other half of the APCs are derived from MHC II$^{-/-}$ bone marrow progenitors), while equally expressing MHC I molecules. Transferring CD4 T cells will trigger spontaneous proliferation, which will differentiate into memory phenotype cells. The pre-existing memory phenotype CD4 T cells would be interacting with MHC II$^{+}$ APCs, while MHC II$^{-/-}$ APCs would remain "untouched" by those memory cells. Freshly transferred naive CD8 T cells undergo spontaneous proliferation even in the presence of functionally competent memory phenotype CD4 T cells only if the half of the APCs does not express MHC II (**Figure 3A**). We also examine if memory CD4 T cells inhibit naïve CD4 T cell spontaneous proliferation *via* similar mechanism. Bone marrow chimeras harboring different haplotype MHC II molecules (for example, haplotype H2*b* and H2*k*) are created (**Figure 3B**). Memory CD4 T cells restricted to the H2*b* haplotype are able to limit spontaneous proliferation of naïve CD4 T cells, restricted to the H2*k* haplotype only when all APCs do express both haplotypes. By contrast, spontaneous proliferation is strongly induced when some APCs express the H2*k*, but not the H2*b* (**Figure 3B**). T cells may down-modulate peptide-MHC complexes on APCs, inhibiting T cell responses to the same peptide-MHC complexes (42). We found that memory T cell interaction with especially DCs induce the production of IL-27 from CD8$^{+}$ DC subsets, and that IL-27 plays a central role in regulating spontaneous proliferation of naïve CD4 and CD8 T cells, because IL-27R$^{-/-}$ naïve T cell

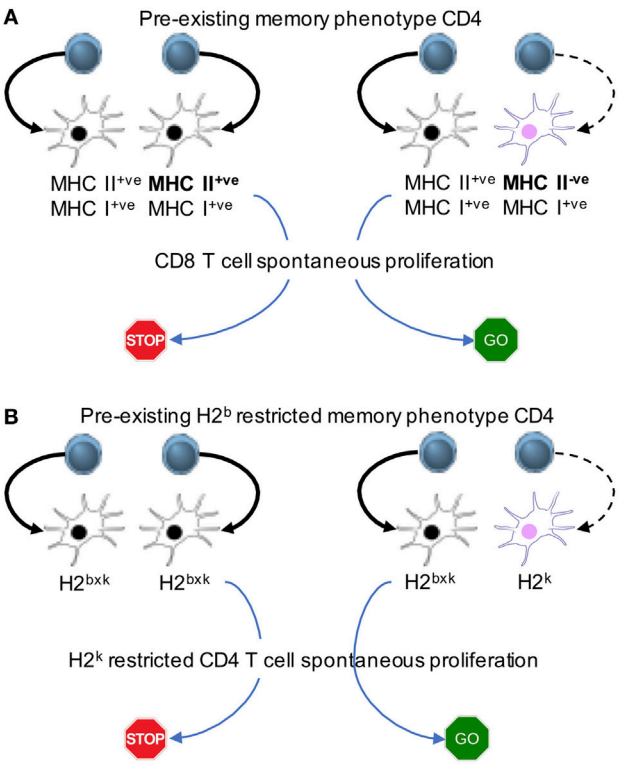


**FIGURE 3** | Memory cell-induced regulation of spontaneous proliferation operates *via* antigen presenting cells (APCs). Mixed bone marrow chimeras in which different APC populations expressing MHC I and II or MHC I alone **(A)** or expressing different MHC II haplotypes **(B)** are created. The first cohort of CD4 T cells is transferred to generate "pre-existing" memory phenotype cells. The second cohort of naive CD8 **(A)** or naive CD4 **(B)** cells is transferred into the recipients. The model system uncovers that the interaction between APCs and pre-existing memory phenotype CD4 T cells is essential to limit the proliferation of new naive T cells.

spontaneous proliferation is not affected by the presence of memory phenotype CD4 cells with complex repertoire diversity and of fully competent APCs (23).

## SPONTANEOUS PROLIFERATION IN A PHYSIOLOGIC SETTING

Does spontaneous proliferation occur in a physiologic setting? We previously reported that naïve T cells transferred into wild type newborn mice undergo spontaneous proliferation (10). During postnatal life, the peripheral lymphoid tissues are rapidly occupied by recent thymic emigrants (43). Those early emigrant T cells are highly proliferating cells as determined by BrdU incorporation and differentiate into memory phenotype cells (44). In fact, delaying the T cell transfer at different postnatal life greatly diminishes spontaneous proliferation of the T cells, suggesting a competition from endogenously generated T cells (10). What is the immunological significance of these spontaneously generated memory phenotype cells? Recent report from Kawabe et al. elegantly demonstrated that these memory phenotype cells acquire T-bet transcription factor expression in response to endogenously supplied IL-12 (38). Most importantly, this study also showed that these IFNγ-producing memory phenotype cells provide a nonspecific host resistance against Toxoplasma infection, enhancing the adaptive immune responses (38). These cells resemble "virtual memory" CD8 T cells expressing foreign antigen-specific memory phenotype in unimmunized animals that are generated by homeostatic mechanisms (45, 46).

## OUTSTANDING QUESTIONS

In conclusion, I would argue that spontaneous proliferation is a key homeostatic process by which endogenous memory phenotype cells are generated and their repertoire complexity increases. There are several key questions worth re-visiting. First, we know very little on the molecular pathways that activate naive T cells to support their differentiation into memory phenotype cells. Although there have been earlier studies examining signaling mediators involved in T cell proliferation within lymphopenic environments, the precise signaling cascade underlying spontaneous proliferation remains poorly understood. What are the key kinases activated during spontaneous proliferation and are they different from cognate antigen-induced activation or homeostatic proliferation? Second, little is known about the antigens that trigger spontaneous proliferation. Are there specific commensal microbe antigens supporting proliferation? Which self-antigens are capable of inducing spontaneous proliferation? Is TCR strength against self-antigens reflected by CD5 expression playing a role in inducing spontaneous proliferation? Last, do Foxp3$^+$ regulatory T cells undergo spontaneous proliferation and is the repertoire complexity of regulatory T cells similarly completed through spontaneous mechanisms seen in conventional T cells? Given the self- or commensal-antigen-driven nature of spontaneous proliferation, it is likely that autoimmunity or inflammatory responses in the intestinal tissues may be induced by dysregulated spontaneous proliferation.

## PERSONAL NOTE

From June 2000 to October 2014, I have had the privilege of working in the Laboratory of Immunology of the NIAID under the guidance of Bill. One day after I joined the lab, he asked me a question. "Will T cells divide in newborn mice ?" Since my Ph.D. project was about neonatal tolerance, his question sounded something testable (and most importantly doable). So, I did the experiment, which changed my interest in Immunology since then. I became fascinated by the concept of lymphocyte homeostasis, which I would still call the main backbone of my current research program. The fact that I was the only fellow in the lab who does not work on IL-4 or on Th2 differentiation did not bother me at all. I still remember how much I had enjoyed doing experiments day and night, preparing and analyzing data for the weekly meeting, discussing the data with him, and planning the next experiments. Nurturing and supportive environment at the Laboratory of Immunology, other faculty members I often interacted with (Ethan Shevach, Ron Germain, Alan Sher, etc.) and colleagues (Jeff Zhu, Lily Guo, Hidehiro Yamane, Javier Cote-Sierra, Nobuki Hayashi, Gilles Foucras, Graham Le Gros, Zami Ben-Sasson, Cyndy Watson, Jane Hu-Li, Irena Stefanova, Dragana Jankovic, and Zvi Grossman), and Bill. It was the perfect combination of all. Even after I had left the lab to run my own laboratory, he continued to inspire and teach me. Thank you, Bill and you are immensely missed.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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MINI REVIEW
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# Studies of Mast Cells: Adventures in Serendipity

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Like many of us who had the great fortune to work with Bill Paul, my science life was immeasurably altered by my interactions with him. Although intimidating at first because of his stature in the immunology world, it was soon clear that he not only truly cared about the specific research we were doing together, but he wished to convey to his trainees an approach to science that was open, always questioning, and infinitely fun. His enthusiasm was infectious and after my training with him, despite stresses due to funding and publishing hurdles, I never regretted the path I took. My research took a sharp turn from the studies of adaptive immunity I had planned on pursuing after my fellowship with Bill to a life long quest to understand the wonders of the mast cell, a relatively rare innate immune cell. This came about because Bill's curiosity and expectation of the unexpected allowed him to view, in retrospect, a rather mundane observation we made together involving a non-physiological transformed mast cell line as something that might be really interesting. I have never forgotten that lesson: Look at the data with an eye on the big picture. Sometimes the unexpected is more interesting than predicted results. His example in this regard was incredibly important when as an independent investigator a mistake in mouse sex determination led to unexpected and very confusing data. Yet, these data ultimately revealed a role for mast cells in male-specific protection in experimental autoimmune encephalomyelitis, the mouse model of multiple sclerosis. Bill's influence in immunology is far-reaching and will continue to be felt as those of us who train our own students and post-doctoral fellows pass on his wisdom and approach to scientific research.

**Keywords: IL-4, mast cells, experimental autoimmune encephalomyelitis/multiple sclerosis, sex-dependent response, IL-33/ST2, testosterone**

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## INTRODUCTION

In January of 1983, I arrived at the Laboratory of Immunology, NIAID, NIH, to work as a post-doctoral fellow with Bill Paul. Like most scientists at the time, I was very enamored by the burgeoning revolution in molecular biology and hoped to gain expertise in gene cloning and expression analysis in the context of the very strong cellular immunology environment of Bill's laboratory. As Bill and I discussed projects, it became clear that we needed a better way to study IL-4, a cytokine then referred to as B cell stimulatory factor-1 or BSF-1. This molecule had been recently identified by Maureen Howard and Bill as an "activity" in phorbol ester-stimulated EL4 T cell lymphoma supernatants that induced B cell proliferation (1). Only by cloning the gene encoding this molecule and having the ability to express reasonably large amounts of pure protein could we accurately determine its regulation and range of biological activities.

The task was daunting for a number of reasons: the enzymes available at the time to carry out reverse transcription were inefficient and made the synthesis of a full-length cDNA a challenge. In addition, our ability to screen for IL-4 activity was dependent on a cumbersome B cell co-stimulatory assay in which purified resting B cells are co-incubated with anti-IgD and a source of IL-4. But in consultation with Ron Germain, our resident expert in all things related to genes, we came up with a plan. I would isolate mRNA from activated EL4 T cells, size fractionate the nucleic acid, subject each fraction to *in vitro* translation using *Xenopus laevis* frog eggs, and test the protein in the B cell co-stimulatory assay. Positive fractions would be used to create a cDNA library. I ordered a colony of frogs, harvested eggs, injected RNA fractions then incubated the eggs overnight, added the egg supernatants to purified low density B cells that were co-stimulated with anti-IgD, and finally measured proliferation using a $^{3}$H-thymidine incorporation assay (2). After seemingly endless negative results (and embarrassing to me, multiple weekly meetings with no good data to present to Bill), one fraction showed activity and this was used as a template for a cDNA library. Unfortunately our hopeful results coincided with two reports that the gene encoding IL-4 had been identified (3, 4). Given the promise of our cDNA library, I quickly identified a clone. The race was on to determine what regulates the expression of IL-4 in normal T cells.

## SERENDIPITOUS DISCOVERY # 1: NOT ALL T CELLS EXPRESS IL-4 BUT MAST CELLS DO

Surprisingly, with the exception of EL-4 cells, none of the long-term T cell lines in the Laboratory of Immunology were positive in our Northern blot analyses, thereby suggesting that there are either unique T cell activation requirements for IL-4 expression and/or there is selectivity in the types of T cells that can express IL-4. Indeed, both of these possibilities turned out to be true. Not long afterward, Mossman and Coffman published their seminal paper revealing the existence of distinct CD4$^{+}$ T helper (Th) cell subsets based on cytokine-producing potential and showed that there is a reciprocal expression pattern of IL-4 and IFN-γ in Th2 and Th1 cells, respectively (5). Subsequent studies have shown that the cytokine microenvironment of a naive CD4$^{+}$ T cell undergoing priming dictates its initial differentiation fate [reviewed in Ref. (6)]. Although frustrating, the lack of an IL-4 response in T cell lines prompted me to take advantage of the unique access to the plethora of biological materials available at the NIH. I canvased other laboratories and collected multiple cell lines representing many distinct lineages and screened them for IL-4 mRNA. Only a subset of transformed and IL-3-dependent mast cell lines was positive.

## A Paradigm Shift in Thinking About Mast Cells' Contributions to Health and Disease

This discovery was published in *Cell* in 1987 (7) and while in retrospect the study was extremely limited and descriptive, Bill immediately recognized the importance of the observation. At the time, studies in mast cell research were largely dictated by adherence to an old paradigm. That is, mast cell activation, mediated solely through FcεR1 cross-linking, elicits the local and immediate release of preformed pro-inflammatory mediators contained in granules. These include lysosome enzymes such as β-hexoseaminidase and cathepsin, biogenic amines such as histamine and mast cell-specific proteases, for example, tryptase and chymase, many of which are involved in eliciting the allergic response. The finding that mast cells expressed cytokines, particularly IL-4, initiated a paradigm shift. Not only could mast cells participate in the effector phase of allergic responses but given they could possibly express this cytokine at low levels without activation, these cells have the potential to directly drive IgE production by B cells.

This accidental discovery of IL-4 production in an unexpected cell type was just the beginning of a massive shift in our ideas about mast cells in health and disease, ideas that had changed only incrementally since the discovery of these cells in late 1800s (8). Subsequent studies by Marshall Plaut, Robert Seder, and Achsah Keegan in Bill's laboratory not only demonstrated that IL-4 production is induced in activated non-transformed lines after IgE receptor cross-linking, but that activated mast cells are also a source of other cytokines, both in culture and *in vivo* (9–11). They also revealed that IL-3 priming significantly increases cytokine production by IgE-stimulated mast cells (12). Since the 1990s, there has been an explosion of data revealing both protective and pathologic roles for mast cells heralding in a new age in mast cell biology [reviewed in Ref. (13)]. Many IgE-independent modes of mast cell activation have since been described. Furthermore, both human and rodent mast cells [foreskin-derived or bone marrow-derived mast cell (BMMC) lines] express a surprisingly large number of cytokines and chemokines under multiple activation conditions. *In vivo*, mast cells have ultimately been shown to affect the outcome of many infections, autoimmune diseases and even cancer. Unexpectedly perhaps, in view of the large amount of evidence that support a pro-inflammatory role, it is now clear that mast cells also have regulatory functions and can suppress damaging immune responses.

## Mast Cell-Deficient Mice: A Key to Deciphering *In Vivo* Contributions of Mast Cells

But this re-imagining of a more widespread role of mast cells was not without controversy. Indeed, a paper published in 2011 by Hans Rodewald and colleagues (14), as well as subsequent work by this group (15) called into question the many reports demonstrating the contributions of mast cells in IgE-independent diseases. This controversy arose in part because there are still no perfect mast cell-deficient mice, although some have fewer non-mast cell defects, thus are arguably better and easier to work with.

One of the earliest descriptions of mast cell-deficient mice came in 1973 by Kitamura and colleagues (16). These mice, designated ($Kit^{W/W^{v}}$), are the result of a cross between mice with two distinct naturally occurring mutations, *W* and *W$^{v}$*, in *Kit*, a gene encoding c-kit, the stem cell factor receptor. Unlike most hematopoietic cells that require c-kit signaling only in early development, mast cells depend on strong and sustained c-kit

signals for their development and long-term survival. WBB6 *Kit*$^{W/Wv}$ (WB *Kit*$^{W/+}$ X C57BL/6 *Kit*$^{Wv/+}$)F1 mice exhibit an 80–90% reduction in c-kit signaling. While this reduced level of activity is sufficient to support the differentiation of most hematopoietic cells, mast cell development is profoundly affected. These mice are also infertile, anemic, neutropenic, have loss of melanocyte pigment production, and show defects in intestinal mobility.

Despite these issues, *Kit*$^{W/Wv}$ mice became the gold standard for *in vivo* mast cell function studies for a period of time. Mast cells can be selectively reconstituted by systemic or local transfer of wild-type BMMCs. If a phenotype is altered in *Kit*$^{W/Wv}$ mice and reconstitution restores it to a wild-type state yet fails to correct the inherent anemia or neutropenia, the phenotype is designated as mast cell-dependent. Mast cells were subsequently implicated in asthma, experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis (MS), arthritis, bullous pemphigus and wound healing, intestinal nematode expulsion, and protection from bacterial infections and protection from animal venoms using *Kit*$^{W/Wv}$ mice [reviewed in Ref. (17)].

Other mice with distinct mutations in *Kit*, such as *Kit* $^{W\text{-}sh/W\text{-}sh}$ mice, have also enjoyed relatively widespread use because unlike *Kit*$^{W/Wv}$ they are on a pure C57BL/6 background, are fertile and are not anemic (18). However, these mice exhibit neutrophilia as well as increased numbers of mast cell precursors and basophils. To circumvent the problems associated with *Kit* mutations, a variety of *Kit*-independent mast cell-deficient mice have now been generated. Because a selective mast cell-specific promoter has not been identified, the approach has relied on Cre recombinase expression under the control of mast cell gene "associated" promoters. Some examples: in *Cpa3*$^{Cre+/-}$ mice, the so-called "Cre-master" mice, in which high Cre recombinase expression is driven by the *Carboxypeptidase 3* (*Cpa3*) promoter, both mast cell and basophil populations are deleted due to Cre recombinase toxicity (14). *Mast cell protease 5* (*Mcpt-5*)*-Cre* mice were crossed to *R-DTA*$^{fl/fl}$ mice resulting in diphtheria toxin produced only by Cre-expressing cells (19). These mice lose peritoneal and ear mast cells and >90% of abdominal and back skin mast cells. However, mucosal mast cells are less affected. *Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice were generated by crossing *Cpa3-Cre* mice with mice containing a floxed *Myeloid leukemia sequence 1* gene and exhibit a 92–100% reduction of mast cells in all sites tested with the exception of the spleen (20). They also are anemic, have neutrophilia, and show a dramatic reduction in basophils in the bone marrow and blood.

## Mast Cells Amplify Central Nervous System (CNS) Autoimmune Disease in Female C57BL/6 and SJL Mice

Our laboratory has exclusively used the *Kit*$^{W/Wv}$ mouse to interrogate the role of mast cells in EAE, a rodent model of multiple sclerosis (MS). MS is an autoimmune demyelinating disorder that develops when myelin-reactive Th1 and Th17 cells gain access to the brain and spinal cord through the normally restrictive blood–brain barrier (BBB) (21). Here, they orchestrate inflammatory damage to the nerve-insulating myelin sheath and the nerve axons. The loss of proper nerve conduction leads to neurological dysfunction that can range from muscle weakness and spasm to loss of motor function and cognitive defects. The most common course of disease is relapsing-remitting MS in which symptoms are intermittent. It is still unclear why MS patients generate pathogenic self-reactive myelin-specific T cells; thus, this autoreactive immune response must be recapitulated in mice by active immunization with myelin, myelin-derived peptides or through adoptive transfer of myelin-specific T cells from immunized mice that are expanded in culture under Th1- or Th17-polarizing conditions (22). Not all mouse strains are susceptible to disease, but $MOG_{35-55}$-immunized C57BL/6 and $PLP_{139-151}$-immunized SJL mice are commonly used as models of chronic and relapsing-remitting disease, respectively.

## Why Mast Cells in EAE/MS?

Our original studies in EAE were prompted by many reports consistent with mast cell involvement in disease. Mast cells are most often associated with blood vessels and nerves and are present in the brain, where they are most numerous in thalamus and hippocampus (23, 24). In addition to their ability to express many mediators including TNF, IL-6, and IL-1β, that promote the pathogenic immune response in MS and EAE, mast cells can also directly provoke demyelination *in vitro* suggesting a potential direct action on myelinated nerves (25). Mast cells are present in the demyelinating lesions of MS patients as are transcripts encoding the mast cell-specific protease, tryptase, as well as histamine and FcεRI (26). Tryptase and histamine are also detected in the cerebral spinal fluid of some patients (27, 28). Drugs that block mast cell degranulation (e.g., proxicromil), or deplete mast cell granules (e.g., cyproheptadine, a serotonin receptor antagonist) inhibit EAE as does hydroxyzine, a histamine receptor antagonist (29, 30).

## Mast Cells Amplify Disease Severity in EAE

In initial experiments, we observed that female *Kit*$^{W/Wv}$ mice on the C57BL/6 and SJL backgrounds exhibit attenuated disease, a phenotype that is associated with decreased inflammatory cell infiltration to the spinal cord and brain. Selective restoration of the meningeal mast cell population *via* BMMC reconstitution is sufficient to restore wild-type disease severity and immune cell influx to the CNS (31, 32). These data indicate that the densely distributed mast cells normally residing in the meninges, a tripartite tissue that surrounds the brain and spinal cord, may be the most relevant population in EAE and MS. In the recent past, the meninges were viewed as merely physical protection for the brain and spinal cord and structures that enclosed the cerebrospinal fluid. This concept has dramatically changed, however, due to several recent discoveries: (a) lymphatic vessels are present in the meninges and provide a passageway for CNS-derived cells and molecules to access the draining deep cervical lymph nodes (33, 34); (b) T cells normally transit through the meninges as part of normal immunosurveillance of infectious microbes that threaten the CNS (35, 36); and (c) many innate immune cells, including macrophages, dendritic cells, and Type 1, 2, and 3 innate lymphoid cells, are permanent residents of these tissues, suggesting this is an immune barrier site analogous

to the skin, gut, and airway mucosa (37–39). Mast cells are relatively prevalent in the dura mater, the outermost layer of the meninges, and in the pia mater, the meningeal layer that lies directly on the brain and spinal cord parenchyma. Of note, mast cells have established roles in regulating vascular permeability in peripheral tissues and in the pia mater are found in close proximity to blood vessels that transition to become the restrictive BBB vasculature. Mast cells are activated within a day of active and passive disease induction and express several mediators including IL-1β, TNF, histamine, matrix metalloproteases (MMPs), CXCL1, and CXCL2 that collectively amplify inflammation and disease severity (40, 41). Among their actions, mast cells contribute to neutrophil recruitment to the meninges and CNS. This neutrophil influx is required for altering BBB integrity and lesion initiation (42, 43). MMPs likely also affect BBB integrity by acting at the glia limitans to degrade the extracellular matrix, a function assigned to mast cells in a model of stroke (44). It has been proposed that meningeal inflammation regulated by mast cells initiates disease by allowing immune cell access to the CNS (37).

Among the most surprising actions of mast cells is their ability to "license" T cells for encephalitogenicity. Primed myelin-specific T cells are not inherently pathogenic but acquire this ability during transit from the secondary lymphoid organs to the CNS. For example, genes that assist in transendothelial migration are induced in T cells post-priming as they transit through the lungs (45). T cells in the meninges can be reactivated by myelin-bearing antigen-presenting cells (36), and it is here that T cells acquire the ability to produce GM-CSF, a cytokine essential for EAE initiation (46–48). In the CNS, GM-CSF$^+$ myelin-reactive T cells recruit CCR2$^+$ monocytes, the major participants in myelin destruction (49). Using an adoptive transfer model of EAE, we demonstrated that T cell-mast cell cross talk in the meninges is crucial for T cell pathogenicity (50). As a result of these interactions, mast cells express IL-1β, which acts on T cells to elicit GM-CSF. In the absence of mast cells or if mast cells are unable to express IL-1β, GM-CSF production is reduced, as is EAE severity.

It is still unclear how this cellular cross talk is initiated, although there are reports of mast cell-T cell interactions through mast cell MHC class II expression (51, 52). Others have shown that direct interactions between for example, OX40/O40L, trigger both mast cell and T cell activation suggesting a contact-dependent mechanism mediates this cross-activation (53). Finally, a recent report describes mast cell–T cell interactions promote increases in T regulatory (Treg) cell numbers in the lung draining lymph nodes in a model of allergic inflammation (54). Mast cell-T cell co-culture experiments demonstrated that mast cell-derived IL-2 was critical for this Treg cell expansion.

## SERENDIPITOUS DISCOVERY #2: A CONTEXT-DEPENDENT ROLE FOR MAST CELLS IN EAE: SEX MATTERS

Until recently, all of our studies to interrogate the pathologic role of mast cells were performed using female mice. This was particularly relevant in the SJL strain because male SJL mice develop little or no disease. However, an incident of inaccurate sex determination in young mice resulted in our accidental analysis of a cohort of wild-type and *Kit*$^{W/Wv}$ males. Although it took some time to sort out, we observed that the *Kit* mutation, rather than protecting as it does in females, causes significantly worse disease in males. This unintentional finding ultimately led to surprising insight into the cellular and molecular basis of sex-dimorphic EAE susceptibility.

### Sex-Dependent Protection in EAE

Considerable efforts have been made to understand sex-dependent EAE differences in SJL mice because they provide a model of the profound differences in MS susceptibility that exist in humans where females show a threefold to fourfold higher incidence than men (22, 55–57). Several studies have demonstrated that protection in SJL males is not due to a lack of an anti-myelin response but rather to qualitatively distinct T cell responses: whereas females generate a pathogenic Th17 cell response, a non-harmful Th2 response dominates in males (58, 59).

We observed that male SJL *Kit*$^{W/Wv}$ mice generate a Th17 anti-myelin response consistent with their clinical disease (60). Mast cell reconstitution does not restore protection in *Kit* mutant males indicating these cells are not sufficient for protection and that another c-kit$^+$ cell is likely involved. Indeed, further analysis of these mice revealed an additional c-kit-dependent phenotype. Type 2 innate lymphoid cells (ILC2s) express c-kit and are also in deficit in *Kit*$^{W/Wv}$ mice. ILC2s are CD45$^+$, Lineage$^-$IL-7Rα$^+$ innate immune cells. They are distinguished from other members of the ILC family including ILC1s and ILC3s based on their expression of Th2 cell lineage determining transcription factors (GATA3$^{high}$ and RORα$^+$), ST2, the IL-33 receptor, and their production of Th2 cytokines. This was of interest because ILC2s are established players in immunity to parasites and allergic disease, where their expression of IL-13 is essential for robust Th2 responses (61–64). Thus, our observations suggested the possibility that the lack of ILC2s in *Kit*$^{W/Wv}$ males prevented the development of the Th2-dominated response characteristic of male wild-type mice.

These data also raised the possibility that the Th17-dominated response in females is due to absent or dysfunctional ILC2s. Yet female SJL mice have similar steady state populations in the multiple tissues analyzed (bone marrow, lymph nodes, brain, spinal cord, meninges), and there is no difference in the response of wild-type male vs. female-derived ILC2s when provided with activating factors such as IL-33, IL-2, and IL-7 (65). However, there are sex-determined differences in the expression of activating factors, including IL-33. Upon immunization males express significantly higher levels of IL-33 mRNA in the lymph nodes, meninges, brain, and spinal cord. IL-33 is considered the most potent ILC2 activating factor (66), and the importance of this cytokine in disease protection was verified by experiments demonstrating that IL-33 treatment of females prior to disease induction prevents EAE. Importantly, treatment at peak disease reverses clinical symptoms. In both cases, ILC2s are activated and even an established Th17 response shifts to one that is Th2-dominated. Anti-IL-33 treatment of males blocks ILC2 activation and renders the mice susceptible to EAE (65).

## Mast Cells Are Activated to Express IL-33 Upon Immunization

Mast cells are one important source of this cytokine *in vivo* (65). IL-33 mRNA and protein production by mast cells can be detected in the meninges after disease induction. Furthermore, mast cell-deficient males show a significantly reduced IL-33 response upon immunization when compared to wild-type males and BMMC reconstitution partially restores this response. These data have led to a model in which male *Kit*$^{W/Wv}$ mice fail to generate a Th2 response because they lack both an important source of IL-33, mast cells, as well as the IL-33 responder population, ILC2s.

## Testosterone-Induced IL-33 Elicits the Male-Specific ILC2-Dependent Protective Pathway

This model explains the inability to restore protection to susceptible *Kit*$^{W/Wv}$ males with mast cells alone. But what accounts for the male-specific expression of IL-33? Testosterone was a likely candidate. This sex hormone is found at sevenfold to eightfold higher levels in adult males than females, and is associated with male-protection (57, 67). MS susceptibility in men increases with the normal age-related decline in testosterone levels, and limited clinical studies have shown treatment of male patients improves cognitive symptoms and gray matter atrophy (68, 69). In mice, testosterone treatment of females attenuates the pathogenic T cell response and reduces disease. Likewise, testosterone blockade using the androgen receptor (AR) antagonist flutamide confers susceptibility to males (70–72).

Both male- and female-derived peritoneal mast cells as well as BMMCs express the AR (65). However, testosterone induces IL-33 protein and mRNA expression only in male-derived BMMCs. This male-specific expression pattern was also evident with other modes of activation. Stimulation with heat killed *Mycobacterium* (Mtb) or IgE receptor cross-linking induced a relatively robust *Il33* response in male- but not female-derived cells. Taken together, we propose that testosterone induces a cascade of events that lead to the expression of mast cell IL-33, activation of ILC2s, and priming of Th2 responses (**Figure 1**). It is notable that immunized males show increases in serum testosterone over time, with

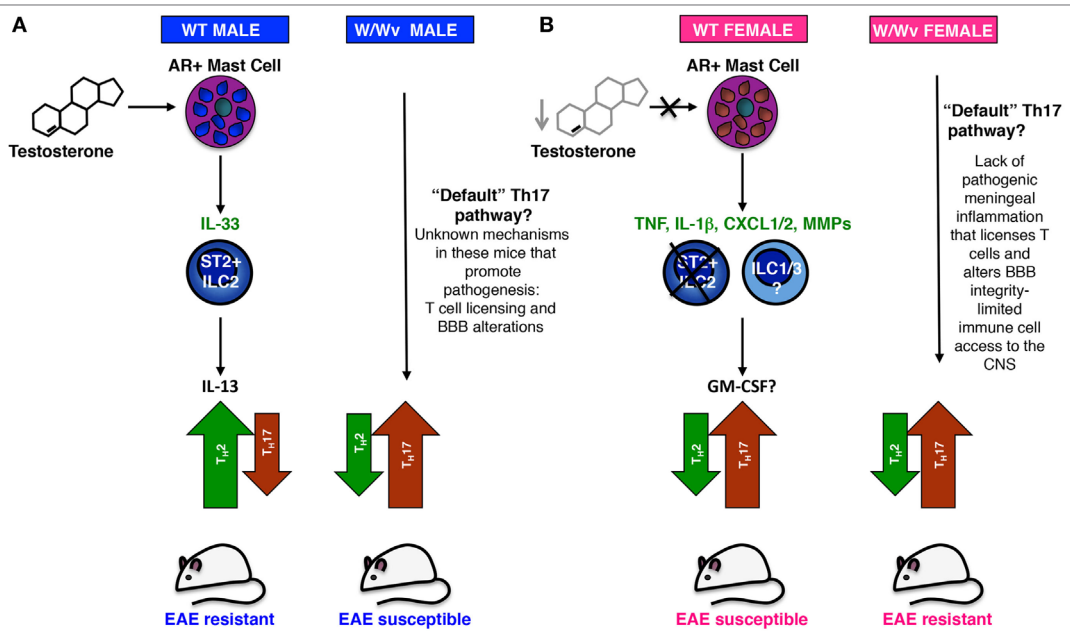


**FIGURE 1** | A model of sex-dimorphic T helper (Th) responses in experimental autoimmune encephalomyelitis (EAE) informed by studies in *Kit*$^{W/Wv}$ mice. **(A)** Testosterone-dependent IL-33 production in androgen receptor$^+$ (AR$^+$) mast cells promotes a non-pathogenic Th2 anti-myelin response in $PLP_{139–151}$ immunized wild-type SJL males. Early IL-33 production by mast cells (and perhaps other AR$^+$) cells activates ST2$^+$ innate lymphoid cells (ILC2s), which in turn express IL-13, a cytokine that polarizes the response to one that is Th2-dominant. This Th2 polarization appears to take place during priming in the secondary lymphoid organs and is likely maintained in Th2 effector cells by resident ILC2s in the meninges and Central Nervous System (CNS). Testosterone potentially acts in two ways: (1) acute increases in systemic testosterone directly activate mast cells, and perhaps other AR$^+$ cells, resulting in increased IL-33 expression; (2) long-term testosterone exposure may also exert effects on the *Il33* chromatin landscape in mast cells, enabling higher potential for activation-induced expression. In the absence of IL-33-producing mast cells and ILC2s, a major but not exclusive IL-33 target cell, male *Kit*$^{W/Wv}$ mice cannot generate a strong Th2 response and "default" to a pathogenic Th17 response. In addition to anti-myelin-specific Th17 cells, unknown mechanisms promote inflammatory cell influx to the CNS and promote disease susceptibility in these mice. **(B)** Immunized wild-type females "default" to a Th17 response because they lack sufficient testosterone to elicit the IL-33–ILC2–Th2 pathway. Low testosterone may fail to acutely induce IL-33, but may also affect the *Il33* chromatin landscape, lessening the potential for mast cell IL-33 expression. Upon activation female-derived mast cells express an alternative set of more pro-inflammatory effector molecules. IL-1β-producing mast cells "license" these T cells as they transit through the meninges by eliciting GM-CSF production and enhancing encephalitogenicity. Inflammatory cell influx to the CNS is facilitated by mast cell TNF, CXCL1/2, and matrix metalloprotease (MMP) production that recruits neutrophils and degrades the extracellular matrix, altering blood–brain barrier (BBB) integrity. Increased ILC1 and ILC3 activity in females may also facilitate meningeal inflammation and immune cell infiltration to the CNS (73). Resistant female *Kit*$^{W/Wv}$ mice also generate a Th17 response, but in the absence of meningeal inflammation and T cell licensing, driven by mast cell-derived TNF, IL-1β, CXCL1/2, and MMPs, these cells have only limited access to the CNS parenchyma.

levels peaking at ~ day 13 post-immunization. We speculate that (a) inflammation enhances the male hormonal milieu, which in turn further promotes a shift to Th2-mediated protection, and (b) females do not express the threshold level of testosterone needed to activate this pathway.

## The Ever-Evolving View of Mast Cells—We Must Leave the Paradigms Behind

There are several implications of these data in addition to the obvious possibilities for new therapeutic approaches to neuroinflammatory diseases. First, they further demonstrate that mast cells respond in a context-dependent way. While this concept is not new when considering distinct tissue-specific actions of mast cells, we show that the hormonal context can radically alter outcomes of mast cell activation in cells derived from the same tissues. Indeed, in addition to sex-specific responses by meningeal mast cells in immunized mice, there are distinctions in BMMC responses in culture. This is strikingly illustrated by the fact that female-derived BMMCs do not express appreciable IL-33 even when stimulated with Mtb or through IgE receptor cross-linking (65). Rather these modes of activation induce *Tnf* and *Il1b*, genes that are not as highly expressed in male-derived mast cells. It is likely that in addition to acute influences on mast cell activation, the hormonal environment shapes the overall potential for gene expression in these cells by altering chromatin accessibility. The directive from the NIH director that sex must be considered as a biological variable has not come too soon.

Our findings add to the growing evidence that mast cells can serve protective roles in some settings. Evidence showing that mast cell–Treg cell interactions can be important in limiting inflammation also continues to accumulate. Mast cells appear to act downstream of Treg cells in an allograft tolerance model in which mast cells are required for prolonged survival. It is proposed that IL-9 production by Tregs activates local mast cells to produce IL-10 to limit rejection (74). In a papain-induced model of allergic inflammation, mast cells act upstream of Tregs (75). In this scenario, IL-33, presumably passively released after protease damage of lung epithelial cells, elicits IL-2 production by mast cells. IL-2 promotes Treg cell expansion and limits the damaging effector response mediated by eosinophils. In view of these studies and given the reported protective IL-33-dependent role of a subpopulation of ST2$^+$ Tregs in a model of inflammatory bowel disease (76), it will be important to understand whether these and other ST2$^+$ cells are targets of this mast cell produced cytokine in EAE/MS. Mast cells limit inflammatory damage in a Treg-independent manner as well. Not only do mast cell proteases degrade animal venoms and can decrease the pathological responses associated with envenomation (77), IL-10 and IL-2 produced by mast cells limit chronic inflammation in models of contact sensitivity (78, 79) and in a graft-versus-host disease model where mast cell-derived IL-10 is required for prolonging graft survival (80).

## Are Mast Cells Really the Master Cell?

As alluded to above, results generated using *Kit*$^{W/Wv}$ mice have been called into question because they are often not replicated when *Kit* $^{W\text{-}sh/W\text{-}sh}$ or *Kit*-independent mast cell-deficient mice are used [discussed in beautiful detail in Ref. (17)]. A stunning example is the multitude of papers using Cre-master mice to demonstrate that mast cells are dispensable in many settings where mast cells were previously shown to make a contribution [reviewed in Ref. (15)]. The original report by Rodewald's group showed that anaphylaxis and expansion of intestinal mast cells in a *N. brasiliensis* infection model are extinguished in *Cpa3*$^{Cre+/-}$ mice, supporting the validity of using these mice to assess mast cell contributions in responses in which IgE-activated mast cells are the major effector cells (14). However, unlike previous (but not all) EAE studies by us and by others using *Kit*$^{W/Wv}$ mice suggesting mast cells exacerbate disease (81), *Cpa3*$^{Cre/+}$ mice are fully susceptible to EAE. The reasons for these differences are still unclear, but there are several possibilities: Mast cells provide an accessory function that can amplify or lessen a response mediated by activated T and B cells. In cases where strong T or B cells are induced, the more subtle contributions of mast cells may be masked. Evidence that altering the EAE disease induction protocols affects the ability to assign a mast cell contribution comes from multiple laboratories using the same *Kit*$^{W/Wv}$ mice (81). Of note, the strong disease induction conditions used in the Cre-master mouse study (corroborated by high morbidity in all groups) also support this alternative interpretation of the data. Age of mice and environment, including differences in microbiota, are also variables that may affect disease severity.

So what do we make of all the data that comes from *Kit*$^{W/Wv}$ mice? The dramatically different mast cell functions revealed by our analyses of male and female *Kit*$^{W/Wv}$ mice in EAE confirm that, under the right experimental conditions, *Kit* mutant knock in mice are valid tools to delineate the role of mast cells and other c-kit$^+$ cells in disease models. While we still need more selective ways to genetically deplete mast cells, the data generated from studies with *Kit*$^{W/Wv}$ mice should not be discarded out of hand: in females, the lack of mast cells resulted in reduced clinical disease, which is restored to wild-type severity with reconstitution. Although disease scoring is too often subjective, more objective assessments revealed the alteration of several mast cell-dependent pathways that amplify inflammation. These include meningeal mast cell activation, neutrophil influx to the meninges, BBB breach, inflammatory cell influx to the CNS, mast cell IL-1β expression in the meninges, and acquisition of T cell GM-CSF production. Importantly, our use of male *Kit*$^{W/Wv}$ mice revealed a pathway that could not have been easily identified in other *Kit*-independent mast cell-deficient mice. Mast cell reconstitution failed to confer protection to *Kit*$^{W/Wv}$ males, indicating mast cells alone cannot restore the male-specific wild-type phenotype. Thus the system worked, as it should. Indeed, these experiments allowed us to identify the deficit in c-kit$^+$ ILC2s in *Kit*$^{W/Wv}$ mice and to assign them as additional critical players in male-specific protection. The role of a c-kit$^+$ pro-inflammatory ILC3 population in EAE exacerbations was also revealed using these mice (38). It is tempting to speculate that c-kit$^+$ ILCs may contribute to other functions assigned solely to mast cells using *Kit*$^{W/Wv}$ mice. That is, the lack of both mast cells and ILCs in *Kit*-dependent mast cell-deficient mice may explain some of the discrepancies observed in studies

using *Kit*-independent mast cell-deficient mice in which ILC populations are likely unaffected.

As alluded to earlier, mast cells have the potential to influence many if not most biological processes in humans due to their widespread distribution in most tissues, their proximity to blood vessels, the seemingly endless variety of effector molecules they can produce, and their ability to interact with both immune and non-immune cells. Indeed, in a review by Rodewald and Feyerbend it was stated, "There is arguably no second cell type in the immune system as powerfully equipped with a large array of chemically diverse and highly potent compounds" (15). Not surprisingly, soon after the realization that mast cells can act outside the realm of allergy the experimental dam broke so to speak, leading to many studies over the years showing mast cells modulate processes far beyond the innate and adaptive immune responses that dictate the outcomes of autoimmunity, cancer, infection and neuroinflammation. Among the perhaps unexpected activities of mast cells are roles in vascular disease (82), angiogenesis and tissue remodeling (83, 84), diabetic wound healing (85), migraine headaches (86), anxiety (87), metabolic syndromes (88), fertility (89, 90), and development of mammary glands (91).

The challenges ahead are many. First, it is important to ultimately delineate the underlying reasons for the conflicting data derived from various experimental models. Second, the observed strain and sex variations in mast cell activity defined in mice indicate that many new paradigms that arose based on studies in one mouse strain or sex must be revisited to take these variables into account. Third, there is likely to be similar and more extensive mast cell heterogeneity in humans. Uncovering these differences will be a daunting task. An ultimate goal may be to target these cells in disease therapy, but in some settings, we will need to understand their actions in each individual context in order to make decisions about whether blocking or enhancing their activation is desirable. Only by keeping our eyes on the big picture, we will continue to gain greater insight into the biology of these amazing cells, cells which I have made my life's passion, all because of Bill Paul.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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# Controlling Mast Cell Activation and Homeostasis: Work Influenced by Bill Paul That Continues Today

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Mast cells are tissue resident, innate immune cells with heterogenous phenotypes tuned by cytokines and other microenvironmental stimuli. Playing a protective role in parasitic, bacterial, and viral infections, mast cells are also known for their role in the pathogenesis of allergy, asthma, and autoimmune diseases. Here, we review factors controlling mast cell activation, with a focus on receptor signaling and potential therapies for allergic disease. Specifically, we will discuss our work with FcεRI and FγR signaling, IL-4, IL-10, and TGF-β1 treatment, and Stat5. We conclude with potential therapeutics for allergic disease. Much of these efforts have been influenced by the work of Bill Paul. With many mechanistic targets for mast cell activation and different classes of therapeutics being studied, there is reason to be hopeful for continued clinical progress in this area.

**Keywords: mast cell, IgE, IgG, IL-4, IL-10, TGF-β, Stat5, allergy**

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## INTRODUCTION

Mast cells were first described by Paul Ehrlich in 1878. The future Nobel Laureate identified them based on their unique staining characteristics with aniline dyes and their position in all tissues of the body, particularly at interfaces with the external environment (1). As with T cells and macrophages, mast cells are a heterogeneous population, consisting of at least two major subsets (2). Although the origin of these cells remained elusive, seminal work in the 1970s and 1980s established that mast cells originate from hematopoetic stem cells in the bone marrow, spleen, fetal liver, and peripheral blood (3–5). Subsequent work showed that mast cells can be differentiated and expanded *in vitro* with relative ease, which greatly increased interest and progress in the field. What followed was detailed work describing how mast cells bind and respond to IgE, providing evidence for the role of mast cells in allergic disease (6, 7).

However, our understanding of mast cell biology changed drastically in the late 1980s with work by Bill Paul and colleagues. Bill Paul's career centered on understanding T cell function and cytokine biology, contributing to the discovery, and understanding of T cell MHC-restriction, the B cell receptor mIg, IL-4, and Th2 polarization, as he eloquently described in a review of his life's work (8). Following the discovery of IL-4, Bill Paul's group showed that transformed and non-transformed mast cells express IL-4 in response to PMA and ionomycin (9) and that mast cells secrete a Th2-like panel of cytokines, including IL-4, in response to IgE receptor cross-linking (10). These were tectonic shifts in our fundamental understanding of mast cells, providing evidence that in addition to granule release, mast cells produce cytokine mediators that influence adaptive immunity and have a broader role in allergic disease. It is in keeping with Bill Paul's visionary abilities that he could abruptly cast a broad light on field tangential to his primary interests. He would go on to publish two dozen mast cell-related articles, including one that initiated our group's focus on Stat5 in mast

cell biology (11). Furthermore, Bill trained many researchers who have gone on to have productive careers in the field of mast cell biology and allergic disease, including the senior author of this article, Takashi Saito, Fred Finkleman, Melissa Brown, Achsah Keegan, and Joshua Milner, many of whom have work cited here. In this review, we will cover several areas of mast cell activation and homeostasis, all of which are of great interest to our lab and have been impacted by Bill Paul's intellect and productivity.

## MAST CELL GROWTH, SURVIVAL, AND APOPTOSIS

Mast cells are long-living tissue-resident immune cells that migrate to and differentiate within the tissue. Development, migration, and survival are shaped by two growth factors, in particular, SCF and IL-3, which are included in **Figure 1**. In healthy tissue, mast cells are maintained in constant numbers, while the mast cell population increases dramatically in chronically allergic tissue (12). This section will summarize findings on mast cell survival and death. Prior to discovery of the c-Kit receptor and its ligand SCF, mice with double mutations at the *ckit*-encoding *W* loci ($W/W^v$ mice) or *scf*-encoding *Sl* loci ($Sl/Sl^d$ mice) were known to exhibit hypoplastic, macrocytic anemia, sterility, and a lack of cutaneous melanocytes (13–15). Importantly, these mice were found to have a defect of mast cells in $W/W^v$ mice due to lineage abnormality and a defect of mast cells in $Sl/Sl^d$ mice due to an abnormality in the microenvironment (4, 16). A decade later, two groups reported that the *W* gene product encodes the c-Kit tyrosine kinase receptor (17, 18), while in 1990, eight groups described and identified the ligand for c-Kit: SCF/MGF/steel factor, encoded by the *SI* locus [prefaced in Ref. (19)]. These papers clarified the complementary receptor–ligand relationship yielding the similar phenotypes of $W/W^v$ and $Sl/Sl^d$ mice and suggested a role for c-Kit and SCF in mast cell development.

c-Kit is a tyrosine kinase growth factor receptor, with a large extracellular domain of five Ig-like domains, a single transmembrane span, and a long cytosolic tail, containing a tyrosine kinase domain and tyrosine phosphorylation sites (21). Dimerization initiates phosphorylation of at least eight tyrosine residues, serving as a docking site for Src homology 2 domains present on signaling proteins such as Grb2, Gab2, Lyn, Fyn, PI3K, phospholipase Cγ, and the negative regulator, SHP-1 (22).

SCF is best known for eliciting mast cell survival and inhibiting apoptosis (23). SCF-mediated activation of the PI3K–AKT cascade is important for mast cell survival, inactivating the pro-apoptotic proteins Bad and Bim, and increasing expression of the pro-survival proteins, such as Bcl-2 and Bcl-XL (24, 25). Interestingly, somatic and germline *ckit* gain-of-function mutations are present in mastocytosis patients (26, 27), suggesting regulatory control by the c-Kit pathway.

In addition to its role in survival, SCF has other important effects. These include inducing mast cell migration, adhesion, and IL-6 secretion (28–30). SCF also augments mast cell activation by FcεRI, ST2, and TLR4, receptors which will be further reviewed below (31–33). c-Kit signaling enhances mast cell degranulation

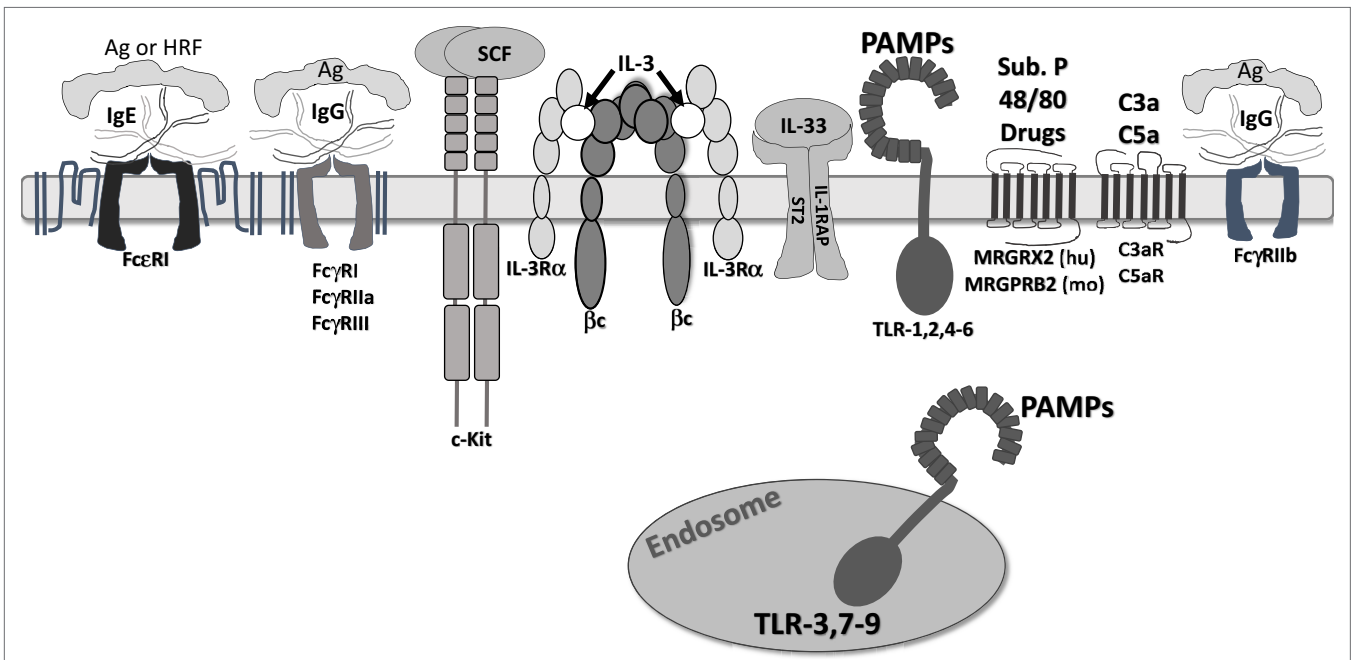


**FIGURE 1** | Receptors that regulate mast cell function. The receptors shown are confirmed to regulate mast cell function. They are depicted at approximate scale. All except FcγRIIb are known to induce mast cell degranulation and/or cytokine secretion. FcγRIIb activates SHIP-1 and SHP-1, suppressing inositol and tyrosine kinase activity. c-Kit is a weak mast cell activator, but augments signals by other receptors. IL-3 receptor is modeled after work by Broughton et al. (20). Note that ligands for Mas-related G protein-coupled receptor-X2 (MRGPRX2)/B2 are not fully known, but include drug classes discussed in the accompanying text. In addition, we do not show "cytokinergic" IgE molecules. These form aggregates in the absence of antigen and elicit FcεRI signaling.

and cytokine production by inducing calcium influx and transcriptional activity (34). These abilities make SCF arguably the most critical factor controlling mast cell biology.

IL-3 is also an important factor for mouse mast cell precursor survival, with an unclear role in human mast cells. Mouse mast cells can differentiate in response to IL-3 (mucosal tissue type) or IL-3 + SCF (connective tissue type), while human mast cells typically require SCF + IL-6 for differentiation (35–38). Contradictory studies suggest that IL-3 plays no role in human mast cell progenitor differentiation (38), while a recent study demonstrated that IL-3 alone is sufficient to drive differentiation and survival of human mast cell progenitors (39). Similar to SCF, withdrawal of IL-3 from cultured mast cells induces apoptosis and appears to play an important role in mast cell survival and development in at least mouse mast cells (23, 40). Lantz et al. showed that IL-3-deficient mice have normal numbers of mast cells in the naïve state, but fail to properly expand intestinal mast cells in response to parasite infection (41).

To balance cell growth, mast cells have pathways for both apoptosis and autophagy. The Fas and TRAIL death receptors are expressed on primary mouse and human mast cells and various mast cell lines (42, 43). Mast cells are susceptible to Fas- or TRAIL-mediated apoptosis *via* caspase activation (1, 43). However, they can overcome Fas death signals by upregulating Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein, a caspase-8 inhibitor that lacks the cysteine domain (42). While transformed and healthy mast cells use this mechanism to bypass Fas-mediated cell death, making this an unreliable and weak apoptosis inducer (43), our group has shown that that BMMCs are more responsive to Fas/FasL-induced apoptosis in the presence of IL-4 and IL-10 (44). Furthermore, rather than acting solely as an apoptosis signal, Fas functions in mast cell development and maturation (45).

In addition to apoptosis, mast cells also undergo autophagy, which controls the clearance and reuse of intracellular organelles and proteins and is essential for eukaryotic cell survival. Light chain (LC)-3 is localized to autophagosomes through conversion of LC3-I to LC3-II, which requires *Atg5* and *-7*. LC3-II-expressing autophagosomes are delivered to lysosomes, where the autocargo is degraded (46). Conversion of LC3-I to IC3-II has been shown to be constitutive in BMMC, in which LC3-II associated with secretory granules (47). The same study showed that BMMC lacking *Atg7* or *12* have normal granule formation, but defective IgE-mediated degranulation, demonstrating the importance of autophagic machinery in granule movement and release. Furthermore, dysregulation of autophagy in mast cells has been shown in various disease states such as systemic sclerosis, chronic rhinosinusitis, and asthma (48–50). Overall, research on mast cell survival, apoptosis, and autophagy pathways suggests that these pathways are important for both maintenance and function in both health and disease.

## ACTIVATING LIGANDS AND RECEPTORS REGULATING MAST CELL FUNCTION

Mast cells respond to myriad signals consistent with their role in defense against pathogens, while contributing to their effects in allergy, asthma, and autoimmunity. In addition, mast cells are a heterogeneous population, particularly susceptible to different tissue microenvironments during development and maturation. Residence in different tissues is linked to distinct mast cell protease and receptor expression (51–53). Moreover, mast cell phenotype is highly "tunable" based on short-term modulation by inflammatory stimuli, growth factors, cytokines, and metabolites (52, 54–56). We will first describe several activating receptors and their stimuli, which are depicted in **Figure 1**.

### IgE and FcεRI

FcεRI, the receptor for IgE, has been the most commonly studied mast cell receptor. While new studies suggest a role for many mediators in allergic disease, IgE remains the best-understood mechanism of mast cell activation in allergic disease (57). The interaction between mast cells and IgE was first shown in 1970 (6, 7). A previously unknown component found in the serum of allergic patients, IgE played a role in the classic Prausnitz–Küstner reaction. In addition, these studies showed that IgE bound to mast cells and basophils. Further characterization found the interaction between IgE and FcεRI, showing that monomeric IgE binds to a singular unit of FcεRI with very high affinity (58). Subsequent studies showed that FcεRI consists of three subunits: one IgE-binding α subunit, one β subunit, and a dimer of disulfide-linked γ subunits (59). FcεRI-mediated activation typically occurs when IgE, bound by its Fc portion (specifically the $C_H3$ domain), interacts with antigen *via* the Fab portion, driving receptor aggregation. This initiates signaling cascades dependent on tyrosine phosphorylation, leading to a biphasic response. The first phase is the immediate degranulation. The second phase is the production of other mediators such as prostaglandins, leukotrienes, cytokines, chemokines, and growth factors (60).

Many of the pathways controlling FcεRI-induced mast cell activation have been described in detail, and we direct the reader to other recent reviews (61–63). However, some aspects of FcεRI activation warrant special attention here. In addition to signaling induced by receptor aggregation, monomeric IgE induces mast cell survival and activation (64–66). Prior to this, there was knowledge that IgE greatly increases FcεRI expression (67, 68), but its ability to induce signals in the absence of antigen was unexpected and controversial. Subsequent studies revealed that the clone of IgE molecule used greatly impacted its effects, with different IgE clones being designated "highly cytokinergic" or "poorly cytokinergic." The latter type has since been shown to form large aggregates through Fv–Fv interactions in the absence of antigen, and hence signals much like IgE + antigen (69). In a similar way, histamine-releasing factor (also called translationally controlled tumor protein or fortillin) binds the Fab region on approximately 25% of IgE molecules tested, allowing for clonally restricted mast cell activation that may be more common among atopic patients (59). These unexpected and clinically important effects of IgE subtypes emphasize the importance of fundamentally understanding receptor–ligand interactions. They also support the approach of suppressing IgE–FcεRI interaction, which is proving effective with the drug omalizumab, discussed in the "Potential Therapies" section below.

## IgG and FcγR

Mast cells can be activated by IgG immune complexes binding pro-inflammatory FcγRI, FcγRIIA, or FcγRIII, which are variably expressed on mouse and human mast cells. These receptors induce a signaling cascade resembling IgE–FcεRI activation that elicits cytokine secretion, arachidonic acid metabolism, and degranulation (70). Our lab has published work suggesting that like FcεRI, FcγR induces Fyn, Lyn, Akt, Erk, p38, and JNK phosphorylation (71–73). Opposingly, the ITIM-containing receptor FcγRIIb activates SHIP-1 and SHP-1, suppressing IgG-induced signals by reducing PI3K and tyrosine kinase activities (74). IgG-mediated mast cell function is less understood than its IgE counterpart, including its direct clinical roles. Various studies show associations for FcγR-induced mast cell function in rheumatoid arthritis, Sjögren's syndrome, multiple sclerosis, bullous pemphigoid, thyroiditis, systemic sclerosis, and glomerulonephritis (74). Thus far, much of these data are correlative. For example, mast cells and their mediators are increased in the rheumatoid synovium (75). Moreover, the results of some studies are contradictory. While there were initial reports of the inflammatory role of mast cells in experimental autoimmune encephalitis [EAE; (76)], there are contradictory reports on the role of mast cells in the disease, potentially due to differences in the severity of the EAE protocols or the mast cell-deficient mouse strains used (77, 78). While many studies have made use of $W/W^{v}$ and $W^{sh}/W^{sh}$ c-Kit-deficient mice, newer mast cell knockout models, such as the CPA3-cre/+ mice cited above and the CPA3-Cre; Mcl-$1^{fl/fl}$, do not have the same changes in neutrophils observed with mutations in c-Kit. Therefore, future studies may clarify the role of mast cells in IgG-associated pathologies such as multiple sclerosis and arthritis (77, 79). This area of research may provide high-impact outcomes, since IgG-induced inflammatory diseases involve pathological processes overlapping with the IgE response and may therefore be responsive to similar therapies.

## Complement Receptors

The complement system is made up of proteolytic pro-enzymes and non-enzymatic proteins that form functional complexes, cofactors, regulators, and receptors (80). Larger fragments derived from C3 and C4 regulate opsonization, phagocytosis, and immunomodulation. The smaller fragments C3a and C5a are anaphylatoxins that mediate inflammatory reactions. Anaphylatoxins can activate mast cells but, like other signals, the outcome depends on factors such as location and microenviroment (81). A few studies have examined the role of complement in mast cell activation. C3a enhances mast cell degranulation in the presence of FcγRI signaling (82), while C5a induces mast cell migration, adhesion, and mast cell mediator production (83).

## Pathogen-Associated Molecular Patterns and Toll-Like Receptors (TLRs)

Toll-like receptors are a part of the pattern-recognition receptor family interacting with a multitude of pattern-associated molecular patterns as well as host-derived damage associated molecular patterns. This family consists of 10 reported receptors (TLR 1–10) (84). Mast cells can express 9 TLRs, including TLR-1, 2, and 4–6 on the cell surface and TLR-3, and 7–9 intracellularly (85). However, some caveats are worth noting: TLR8 has not been detected on human mast cells; receptor distribution varies with mast cell location; and some receptors have only been shown at the mRNA level (86).

Toll-like receptor activation induces cytokine secretion, which can proceed through a DAP12-independent signaling cascade (87). TLR-induced cytokine profiles overlap but have some distinctions. For example, TLR-2 activation led to the production of TNF, IL-6, IL-13, IL-4, and IL-5, while TLR-4 induced TNF, IL-6, IL-13, and IL-1b (88). Interestingly, pre-exposure to TLR ligands suppressed IgE-induced mast cell responses in two mouse models, possibly by transiently reducing FcεRI expression (89, 90). By contrast, simultaneous exposure of human mast cells to various TLR ligands and FcεRI stimulation yielded increased cytokine secretion without altering degranulation (91). This is an area that warrants further study and clarification, since environmental and even laboratory exposure to allergens (e.g., house dust mite extract) is often in the context of TLR ligands.

## Compound 48/80, Substance P, and Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2)

Mas-related G protein-coupled receptor-X2 has drawn increased interest recently. Human MRGPRX2 is one of approximately 50 7-transmembrane domain proteins in the larger Mas-related gene family. It is unique in its apparently selective expression on human mast cells in the $MC_{TC}$ subtype, outside of the dorsal root ganglion (92). While the MRGPRX gene family is restricted to humans and other primates, a mouse ortholog, MRGPRB2 has recently been described (93). Using transgene and knockout approaches, this group showed that MRGPRB2 is similarly restricted in expression to connective tissue mast cells. Both orthologs are functional receptors for compound 48/80 and the neuropeptide substance P. While of interest to those using these well-known mast cell-activating stimuli, the more important point is that MRGPRX2 (and likely its mouse ortholog) responds to other peptides and drugs. For example, MRGPRX2 binds the host defense protein LL-37 (94). Three classes of drugs have been shown to activate MRGPRX2: fluoriquinone antibiotics such as ciprofloxacin, neuromuscular-blocking drugs such as rocurinium, and the bradykinin $B_2$ receptor antagonist icatibant (95). Although much remains to be done in this area, it appears that MRGPRX2 has an important role in the pseudoallergic reactions induced by some drugs. Inhibiting this receptor may therefore be clinically important.

## Chemokines and Their Receptors

Chemokines are cytokines known to induce cellular locomotion. These are particularly important in the migration of cells to areas of inflammation. All chemokine receptors described are seven-transmembrane-spanning G protein receptors (96). Mast cells have been shown to express multiple chemokine receptors, including CCR1, CCR3–5, CXCR1–4, and CX3CR1 (97). These play a significant role in directing mast cell progenitors to the tissues where they mature, a process that is altered by ongoing inflammation (98–100). In addition to migration, recent

studies show that chemokines can induce partial degranulation (100–102).

## Cytokines and Their Receptors

Cytokines act as messengers and modulate many functions including growth, proliferation, and migration. The importance of cytokines to mast cell biology was first shown in the 1980s, when a method to culture mast cells *in vitro* was being determined. Several groups showed that mast cells could be cultured in media from concanavalin A-activated T cells, cloned Ly + 2 inducer T cells, or WEHI-3B tumor cells. Analysis of this media showed the presence of numerous cytokines such as IL-3, IL-4, IL-9, IL-10, and nerve growth factor (1). The precise role of each cytokine is still a topic of research, partly because mast cells are a heterogenous population due to the microenvironment determining mature phenotype (103). This plasticity allows mast cells to alter their phenotype throughout their lifespan, with the phenotypic profile shaped by the cytokine and growth factor milieu they encounter (54). Our lab has been specifically interested in IL-4, IL-10, and TGF-β, which will be addressed below. In addition, the cytokines IL-33 and TSLP deserve specific attention here, due to their ability to activate mast cells and their known role in allergic disease.

IL-33 is an unusual cytokine, in that it is constitutively produced as a pro-form and localized to the nucleus of barrier cells such as keratinocytes, epithelial cells, endothelial cells, and fibroblasts. Its cleavage and release is stimulated by cell damage or inflammation, supporting its classification as an alarmin (104). Mast cells can also secrete IL-33 upon activation with signals such as IgE stimulation (105). IL-33 interacts with a receptor complex composed of T1/ST2 and IL-1RAcP (106, 107), triggering a MyD88-dependent NFκB-activating cascade resembling TLR signaling. Mast cells were among the first lineages shown to express T1/ST2 (108), 7 years before IL-33 was identified (109). IL-33 is a potent mast cell activator, eliciting survival, maturation, adhesion, and cytokine production (15, 106, 107, 110, 111). IL-33 also enhances mast cell responses to IgE (112) and IgG (113). Furthermore, IL-33 injections induce a rapid peritoneal neutrophil influx that requires mast cell-derived TNF secretion (114). IL-33 is now thought to play a significant role in mast cell-associated diseases such allergy, although precise mast cell-restricted functions are not clear and remain to be elucidated (115).

TLSP has some similarities to IL-33, including expression by epithelial and other barrier cell types and constitutive production among some lineages. In 2015, Bill Paul's group published a ZsGreen TSLP reporter mouse, which showed TSLP expression not only in epithelial cells and keratinocytes but also in dendritic cells, basophils, and mast cells (116). TSLP secretion is induced by TLR-type signals, allergen and air irritant exposure, viral and bacterial infection, and trauma (115). TSLP interacts with a complex of TSLP-R and IL-7Rα. TSLP KO mice have reduced mast cell numbers (117), which is consistent with data showing TSLP induces mast cell proliferation through a Stat6- and MDM2-dependent pathway (117). TSLP does not induce mast cell degranulation and alone is a poor inducer of cytokine secretion. However, in combination with IL-1β + TNF, TSLP elicits the release of many cytokines, including IL-5, IL-6, and IL-13 from human $CD34^+$ progenitor-derived mast cells (110, 111). It should be noted that TSLP-induced cytokine secretion has not been shown using mouse mast cells. Hence, while TSLP is clearly relevant to mast cell development and function, further studies should examine differences between mice and humans, which may be important caveats for experimental design.

# IL-4, IL-10, AND TGFβ1 REGULATE MAST CELL FUNCTION AND HOMEOSTASIS

Regarding other cytokine effects, our lab has specific interest and experience studying IL-4, IL-10, and TGFβ effects on mast cell function and homeostasis. These cytokines augment or impair activation by the mechanisms introduced above, but do not directly induce mast cell activation alone.

## IL-4

IL-4, originally termed B cell stimulatory factor-1, is a cytokine primarily known for its role in antibody driven-allergic disease and protection from parasite infections (118). IL-4 was first discovered to induce B cell proliferation during anti-IgM stimulation and to promote isotype switching to IgG1 and IgE (119–121). In addition, Bill Paul's lab and others showed that IL-4 elicits Th2 differentiation from naïve T cells *in vitro*, which subsequently release IL-4 in a positive feedback loop (122, 123). IL-4 signals through IL-4Rα, as part of a heterodimer containing either the common gamma chain (124) or IL13Rα (125). These receptors allow IL-4 to act on non-hematopoietic cells such as intestinal and bronchial epithelial cells and the vasculature to facilitate the protective expulsion of parasites. For details on IL-4 signaling pathways, we direct readers to reviews (126–128).

Unfortunately, IL-4 is also a major contributor to the symptoms observed with allergy and asthma (129–131). IL-4 was the first cytokine shown to be produced by mast cell lines (9), later confirmed to be secreted in response to IgE and lectin activation in human mast cells (132) as well as IL-33 in mouse mast cells (105). Mast cells also respond to IL-4, first reported to increase proliferation of mast cell lines costimulated with IL-3 by Bill Paul's group (133). This work has been supported with data from other mast cell lines, human gut mast cells, BMMC, and in a mouse model of food allergy (9, 134, 135). Culture with IL-4 + IgE for 4–21 days has been shown to enhance FcεRI receptor expression compared with IgE alone on human cord blood, fetal liver-derived mast cells, and BMMC. It also enhances histamine, $PGD_2$, and $LTC_4$, and IL-5 production following IgE receptor cross-linking (134, 136–139). In addition, IL-4 differentially affected mediator release, augmenting Th2-type cytokines (IL-3, IL-5, and IL-13), and downregulating pro-inflammatory cytokines (IL-6 and TNF) in response to IgE receptor cross-linking and Gram-negative bacterial activation (137).

In contrast to its stimulatory effects on mast cells, IL-4 has been reported to suppress c-Kit expression and mast cell development in human fetal liver-derived mast cells grown in SCF in two studies (138, 140). Similarly, we showed IL-4-mediated inhibition of FcεRI and c-Kit expression on BMMC and PMC following 4–21 days of treatment, an effect dependent on Stat6 (141, 142). IL-4 suppressed IL-4, IL-5, IL-6, and IL-13 secretion induced by

IgE crosslinkage, and TNF and IL-13 secretion induced downstream of SCF. Interestingly, our lab subsequently found that IL-4 increases IgG-mediated degranulation and cytokine production in mouse BMMC, involving Stat6 and increased FcγRIIIA protein expression (143). In addition to the role of IL-4 on cellular activation, we found that IL-4 induces apoptosis in developing mouse or human mast cell precursors derived from bone marrow or umbilical cord blood, respectively (144, 145).

The different pro- and anti-inflammatory effects observed by IL-4 are intriguing. Maturation, phenotype, and culture conditions likely play a role in these IL-4 responses, which we also discussed in a recent review (146). Mouse BMMC is considered less mature than human skin, human intestinal, or mouse peritoneal mast cells, which likely contributes to different experimental outcomes. For example, BMMC attain responsiveness to endothelin-1 when cultured in IL-4, while peritoneal mast cells respond to endothelin-1 without IL-4 (147). Similarly, we found that IL-4 induces apoptosis in developing mouse or human mast cell precursors (144, 145), while mature mouse and human mast cells receive survival and proliferation signals from IL-4, which also promotes the $MC_T$ (tryptase-positive) phenotype in human intestinal mast cells (137). Hence, IL-4 effects on mast cells vary with stage of differentiation, with suppressive signals being most overt on developing or less mature mast cells. Future research should experimentally clarify these observations, examining cells at different maturation stages and following both IL-3 and IL-3/SCF differentiation to examine the effects of mast cell phenotype.

## IL-10

IL-10, originally termed cytokine synthesis inhibitory factor, is a homeostatic mediator in many inflammatory diseases. Secreted by macrophages, Th1 and Th2 cells, regulatory T and B cells, and cytotoxic T cells (148), IL-10 is traditionally considered an anti-inflammatory cytokine. It suppresses monocyte MHC II expression (149), dendritic cell maturation (150), and reduces inflammatory cytokine production from monocytes and neutrophils (151, 152). However, IL-10 also has stimulatory effects. It enhances B cell antibody class switching and plasma cell development (153, 154) and increases IL-2-induced proliferation and cytotoxic activity in NK cells (155). These effects correlate with clinical data, as IL-10 therapy has induced platelet loss in RA patients (156) and promoted IFNγ production in sepsis (157) and Crohn's patients (158). In agreement with this, anti-IL-10 therapy has improved SLE measures in a clinical trial (159). While it is likely that anti-IL-10 therapy impacts many cell types *in vivo*, we have studied both the pro- and anti-inflammatory roles of IL-10 in mast cells.

Similar to IL-4, IL-10 is produced by BMMC and affects mast cell survival, proliferation, and function. IL-10 enhances IL-3-mediated growth of mouse mast cells and their progenitors (160–162). Interestingly, when co-cultured with IL-3 and IL-4, IL-10 induces BMMC apoptosis by diminishing Bcl-2 and Bcl-xL expression, in a Stat6-dependent manner (44), and induces apoptosis following IgE receptor cross-linking, a known pro-survival pathway (64).

Early work showed variable responses to IL-10 treatment. IL-10 was shown to induce BMMC expression of mouse mast cell protease (MCPT)-2 (163). Several studies showed inhibition or no change in TNF, IL-6, and histamine secretion following IgE receptor cross-linking and LPS-induced activation in HMC-1, rat peritoneal mast cells, BMMC, and human cord blood-derived mast cells (164–167). Our lab showed that 4-day IL-10 treatment inhibited FcεRI beta chain expression and IgE-induced TNF production in BMMC (168, 169). Recently, we found that while TNF is diminished, IgE-induced degranulation and secretion of other inflammatory cytokines were *increased* by IL-10 after 24-h treatment, through a Stat3–miR-155 cascade that inhibits the negative regulator, suppressor of cytokine signaling-1 (170). These effects were consistent in mouse and human mast cells and in a model of passive systemic anaphylaxis in our study, as well as a mouse model of food allergy used by Clinton Mathias's group (160). How IL-10 suppresses TNF while enhancing other pro-inflammatory cytokines is unknown and may be important for understanding how mast cell function can be tuned. Our interpretation of these data is that while IL-10 has well-established inhibitory roles, it is not monolithic. Instead, stimulatory effects are clear from both clinical and basic research outcomes.

## TGF-β

Our group has also studied the role of TGF-β in mast cell homeostasis. Similar to IL-10, TGF-β is primarily known for its immunosuppressive effects, but pleotropic activities have been reported based on environmental and differentiation factors (171). TGF-β suppresses T cell proliferation and induces Treg differentiation (172, 173), suppresses B cell proliferation and IgG antibody class switching (172, 174), and inhibits macrophage nitric oxide release and TNF translation (175, 176).

TGF-β also alters mast cell development and function. It enhances early mast cell precursor differentiation and increases protease expression, while antagonizing survival of late stage precursors and mature mast cells (177–179). TGF-β elicits mast cell chemotaxis, but can also suppress migration toward SCF (180–182). Our lab has published that TGF-β-1, -2, and -3 inhibit the expression of FcεRI subunits, c-Kit, T1/ST2, and Fcγ receptor chains in BMMC, peritoneal mast cells, and human skin mast cells. In addition, granule formation, degranulation, and IgE-induced cytokine production were reduced by TGF-β (177, 183, 184). Recently, we found that TGF-β-1, -2, and -3 also inhibit IL-33-induced TNF, IL-6, IL-13, and MCP-1 secretion in mouse and human mast cells and suppress IL-33-induced cytokine production *in vivo* (185). Interestingly, a mouse model of lung inflammation suggests TGF-β enhances LPS-induced mast cell IL-6 production, ultimately inducing neutrophil apoptosis and controlling neutrophilic inflammation (186).

As with IL-4 and IL-10, TGF-β effects on mast cells are altered by microenvironment and genetic background. For example, IL-4 and TGFβ1 block the expression and function of the other's receptor, with IL-4 inhibiting TGFβ1-mediated migration and *vice versa* (187). TGF-β1 has divergent effects on C57Bl6/J versus 129/SvJ mast cells. Not only do 129/SvJ BMMCs resist TGF-β1-mediated suppression of IgE-induced cytokine secretion, these BMMCs show enhanced SCF-induced migration in the presence of TGF-β1 (180). We found that matched C57BL/6J and 129/SvJ BMMC cultures have no difference in TGF-β receptor expression, but 129/SvJ BMMC express twofold to threefold greater levels of Fyn and

Stat5 proteins. Since inhibiting the Fyn–Stat5 cascade appears to be important for TGF-β1-mediated suppression, this may convey resistance. In keeping with these BMMC data, we found that human skin mast cells show considerable donor-to-donor variability in TGF-β1-mediated suppression, when measuring IgE-induced cytokine secretion. These donors also showed variable Fyn and Stat5 expression (180). An additional explanation for TGF-β1 resistance is polymorphic TGF-β receptor variation. C57BL/6J and 129/SvJ strains have known polymorphic variations in TGFβR1 between, and similar variations are tied to human cancers (188).

In summary, mast cell development, survival, and function are greatly altered by IL-4, IL-10, and TGF-β. These effects are impacted by microenvironment, since cytokines can act in opposition; by stage of differentiation, since precursors and mature mast cells can respond differently; and by genetic background, with some inbred mouse strains and human donors showing complete resistance or even opposite responses. Understanding how these signals are integrated will provide a coherent approach to mast cell-associated diseases.

## THE ROLE OF STAT5 IN MAST CELL BIOLOGY

The transcription factor Stat5 is expressed ubiquitously and activated by many growth factors and cytokines, including IL-2, IL-3, GM-CSF, prolactin, erythropoietin, thrombopoietin, and growth hormone (189). Stat5 is implicated in immune homeostasis and inflammation, as mice lacking the 110-kb Stat5A/B locus had perinatal lethality and severely compromised immune systems, similar to mice lacking the proteins γc, Jak3, or IL-7R (190).

Our lab was the first to show SCF-induced Stat5-DNA binding activity in mast cells (11). Utilizing the $Stat5^{DN}$ mouse, mutated to have truncated Stat5A and B lacking the N-terminus and able to form teramers but not dimers (191), we showed that BMMC from $Stat5^{DN}$-expressing mice exhibited increased apoptosis and delayed cell cycle progression when cultured in either IL-3 or SCF alone (192). Specifically, we observed reduced expression of the anti-apoptotic proteins Bcl-2, Bcl-(x)l, reduced expression of the cell cycle regulators cyclin A2 and cyclin B1, reduced mitochondrial membrane potential, and greater activation of caspases-9 and -3. *In vivo*, $Stat5^{DN}$-expressing mice were born with normal mast cell distribution, but had near complete loss of tissue mast cells by 12 weeks of age, indicating that Stat5 tetramer formation is essential for regulating mast cell survival. Furthermore, neoplastic mast cells transformed by mutant c-Kit have constitutive Stat5 activation, which can be successfully targeted to inhibit proliferation and survival (193, 194). Together, these results suggest that Stat5 plays a critical role in mast cell survival.

We later reported direct and transient Stat5 activation downstream of FcεRI-mediated mast cell stimulation (195). Stat5-deficient BMMC showed impaired immediate and late phase mediator release in response to IgE-induced stimulation, indicating a critical role for Stat5 in mast cell function. A subsequent study found that Stat5 activation depends on Fyn kinase expression, and that Fyn and Stat5 are physically associated in resting mast cells (72, 73). We also noted that Stat5 co-localizes with FcεRI in antigen-activated mast cells. Interestingly, the absence of Lyn kinase, Gab2, or SHP-1 enhanced FcεRI-mediated Stat5 phosphorylation (72, 73).

In addition to FcεRI, Stat5 has been implicated in other signaling cascades controlling mast cell function. While IL-33 does not appear to activate Stat5, Bill Paul's group showed that IL-33-induced IL-13 secretion required IL-3-mediated Stat5 activation (196). Furthermore, IL-33 elicits a complex between its receptor, ST2/IL-1RAcP, and c-Kit, supporting the finding that IL-33 signaling is enhanced by SCF in mast cells (31). Since c-Kit is a strong Stat5 activator, Stat5 may similarly contribute to IL-33 signaling *via* this pathway. In a separate line of work, Toshio Kawakami's group reported enhanced Stat5 activity tied to increased mast cell numbers in animal models of atopic dermatitis (AD) and lesions in the skin of AD patients (197), suggesting that Stat5 contributes to this disease phenotype.

Stat5A and B are encoded by distinct genes (198–200). While murine Stat5A and Stat5B exhibit 96% sequence similarity and a very similar expression pattern, these isoforms are not completely redundant and have unique biological activity (199). For example, Stat5A is critical for murine mast cell proliferation and survival (201). Our own targeting of Stat5A or Stat5B using siRNA has found that Stat5B has a selective influence over IgE-mediated mast cell cytokine release and SCF-induced migration, with Stat5A being dispensable (72, 73, 180). The idea that Stat5 can directly promote allergic disease is supported by recent work from Joshua Milner's group, who linked a gain-of-function Stat5b mutation to eosinophilia, urticaria, and dermatitis (202). Although no further work was done to study the specific role and functionality of mast cells in these patients, the Stat5 mutation resulted in greater Th2 cytokine production by CD4 T cells, potentially skewing the immune response toward a Th2 response. It should also be noted that increased STAT5b activity, as observed in these patients, was associated with atopic-like skin inflammation that typically involves mast cell activation. Collectively, these findings show that Stat5 is one of the central factors controlling mast cell survival and function. With evidence that Stat5 can be targeted pharmacologically, we see this as a productive avenue for addressing mast cell-associated diseases.

## THERAPEUTICS TARGETING MAST CELLS

Due to the variety of inflammatory diseases in which mast cells participate, targeting mast cell function and survival can be broadly effective. This section will briefly discuss current and potential mast cell-directed therapies. We also refer the reader to several recent reviews focused on this topic (203–206).

There are several therapeutics on the market that are FDA approved for allergic disease or asthma, targeting mast cell activation and/or mediators. These include H1 inhibitors that prevent histamine signaling (207) and antagonists of leukotriene synthesis or signaling *via* the CysLT1 receptor (208). While these drugs are effective in targeting select mediators, broader inhibition of the many mast cell-derived inflammatory factors can be needed for clinical efficacy. We should note here that the newer generation antihistamines have better binding affinity for histamine receptors, reduced adverse side effects, and often have mast cell stabilizing and anti-inflammatory properties in addition

to their effects on histamine, which may provide better relief for patients (209). Corticosteroids such as dexamethasone are effective inhibitors of mast cell activation and a proven treatment for mast cell-associated diseases (210, 211). In fact, we recently found that dexamethasone inhibits not only IgE- but also IL-33-mediated mast cell function (212). However, steroid medications have many adverse effects and reduced efficacy in viral-induced exacerbations, asthmatics who smoke, and in more severe forms of the disease (213, 214). The mast cell stabilizer disodium cromoglycate is approved for some mast cell proliferative and activation diseases (215), although the mechanism of action is poorly understood (216), effects are slower than drugs like antihistamines, and there is evidence that mast cell stabilizers are less efficacious than inhaled corticosteroids for asthma (209, 217). More recently, a humanized anti-IgE antibody, omalizumab, has been developed to prevent binding of circulating IgE to FcεRI and the downstream effects of cross-linking (218, 219). Omalizumab is a common and preferred treatment for chronic urticaria (220, 221) and shows efficacy for the treatment of asthma (218, 219); however, like many drugs, there appear to be responders and non-responders (222). These drugs represent both progress toward suppressing mast cell function and shortcomings supporting further development.

## Repurposing FDA-Approved Drugs

There are several FDA-approved therapeutics with potential to treat diseases caused or exacerbated by mast cell activation (**Table 1**). For instance, the tricyclic antidepressant doxepin was found to be a potent H1 receptor inhibitor and is prescribed to treat chronic urticaria and AD (223). The repurposing approach to targeting mast cells represents a potentially rapid avenue for clinical progress and includes several drug classes.

### Kinase Inhibitors

Imatinib is a chemotherapeutic agent designed to target the BCR–ABL tyrosine kinase common in chronic myeloid lymphoma (224). Although imatinib was designed to target the ABL tyrosine kinase domain, it also inhibits c-Kit kinase activity. Due to this off-target effect, imatinib has been used to treat mastocytosis cases lacking the c-Kit D816V mutation (225). More recently, imatinib has been tested in a clinical trial to treat severe refractory asthma. Patients treated with imatinib had reduced airway hyperresponsiveness and decreased serum tryptase levels compared to placebo (226), supporting broader use of this drug. Unlike imatinib, masitinib was designed as a c-Kit kinase inhibitor and has been used therapeutically to treat canine mast cell tumors (227, 228). It has since entered clinical trials for human mastocytosis and asthma. In a phase 2a clinical trial, masitinib improved the quality of life in 14 of 25 mastocytosis patients for at least 60 weeks (229). It is also in a phase 3 clinical trial to treat severe and persistent asthma in conjunction with corticosteroids (227). An additional kinase inhibitor capable of suppressing c-Kit, toceranib phosphate, is being tested in canines (236). Kinase targeting in mast cell-associated diseases is not limited to c-Kit. The Syk kinase inhibitor R112 has shown mixed results for allergic rhinitis in two trials (231, 232). Similarly, the phosphatidyl inositol 3′-kinase inhibitor idelalisib has shown progress in a phase 1 trial for allergic rhinitis (233). These studies collectively support the approach of targeting kinases activated early in signaling cascades controlling mast cell function.

### Statins

Statin drugs are HMG-CoA reductase (HMGCR) inhibitors designed to reduce cholesterol synthesis (234). These drugs are primarily approved to treat hypercholesterolemia and reduce cardiovascular disease, but have been beneficial in asthma and atopic diseases, albeit with mixed results. For example, a 1-month trial of simvastatin as a monotherapy for asthma showed little benefit (245), but subsequent studies demonstrated positive effects. Most have employed simvastatin or atorvastatin as an adjuvant therapy. Simvastatin has been shown to decrease eosinophils, improve lung function, and promote Treg

**TABLE 1** | Potential therapies for mast cell-associated diseases.

| Drug | Target | Disease | Comments | Status | Reference |
|---|---|---|---|---|---|
| Imatinib | BCR-Abl/c-Kit | Asthma | | FDA-approved for mastocytosis lacking D816V | (224–226) |
| Masitinib | c-Kit, possibly Fyn, and Lyn | Asthma | | In clinical human trials | (227–230) |
| R112 | Syk | Allergic rhinitis | Early stage results promising | In clinical trials | (231, 232) |
| Idelalisib | PI3K | Allergic rhinitis | Early stage results promising | In clinical trials | (233) |
| Statins | HMG-CoA reductase | Asthma | Mixed results, possibly due to varied responses on different genetic backgrounds | Off-label use of drug approved for hypercholesterolemia | (234–237) |
| Etanercept | TNF | Asthma | Early stage trials for severe asthma. Safety concerns noted | Off-label use of drug approved for use in rheumatoid arthritis | (238, 239) |
| siRNA, morpholino oligonucleotides, CRISPR/Cas9 | Many possible: FcεRI, c-Kit, ST2, tryptase, chymase | Mast cell-associated pathology | Most work is in the conceptual stage, with some *in vivo* rodent studies done | Morpholino-based approach for Duchenne muscular dystrophy is approved | (93, 240–244) |

*This table includes the name of the drug, the known targets, and diseases for which research has currently been conducted using the drug. In addition, important comments on the drug and FDA approval and/or clinical trials progress are noted.*

development in mild asthmatics (235, 246). Similarly, atorvastatin decreased sputum inflammatory cytokine levels, suppressed $LTB_4$ production, and improved quality of life scores among mild-to-moderate asthmatics (247–249). A retrospective study found that among severe asthmatics, statins in combination with inhaled therapies had better asthma control in comparison to patients who were not currently taking statins (236). Despite these encouraging findings, meta-analysis studies show that the overall effects of statins in asthma are at the best modest (249–251).

These conflicting outcomes prompted us to study statin effects on mast cells. Our lab found that one drug in particular, fluvastatin, blocked FcεRI-mediated mast cell activation in human and mouse mast cells and reduced passive systemic anaphylaxis in mice (237). However, these effects showed strong genetic influences: BMMC derived from C57BL/6J mice were most sensitive, BALB/c showed intermediate responses, and 129/SvJ mice were completely resistant. Human mast cells cultured from multiple donors showed similar variation. Our data showed that statin resistance was not tied to HMGCR coding polymorphisms, but did correlate with drug-induced HMGCR upregulation. More importantly, geranylgeranyl transferase (GGT), downstream of HMGCR in the cholesterol pathway, appears to be critical for FcεRI-mediated function. These findings suggest that statin efficacy in mast cell-associated disease might be predicted by measuring drug-induced HMGCR expression, and that targeting GGT may be a better means of disrupting mast cell function.

## Targeting Gene Expression

Another promising means of inhibiting mast cell activation is by selectively suppressing gene expression, including FcεRI, c-Kit, histadine decarboxylase, or other mast cell receptors and mediators. There are several approaches that hold promise, with two decades of clinical trials supporting progress. Morpholino oligomers bind mRNA and either block translation or modify pre-mRNA splicing and induce exon skipping (252). This approach has recently been demonstrated in mice showing that morpholinos targeting the FcεRI β-subunit decreased IgE receptor expression and function on mast cells and basophils (240) and was beneficial in treating a mouse model of allergic dermatitis. Morpholino-based therapy is approved for Duchenne muscular dystrophy (243, 244), suggesting that this approach can succeed.

siRNAs are another gene targeting tool, explored in various clinical trials and on the cusp of FDA approval (253). Similar to morpholinos, siRNAs base pair with mRNAs, inhibiting translation or decreasing half-life. Several studies have used siRNAs to diminish mast cell activation or mediator production *in vitro* (93, 241, 242, 254). A critical step for any nucleotide-based approach is customizing targeting and improving cellular uptake. Several approaches are under study, including nanoparticles and lipid-based carriers (255). Progress in this area may also come from excitement surrounding the CRISPR/Cas9 system. Although less vetted in clinical trials than morpholinos and siRNA, the specificity and efficacy of CRISPR/Cas9 elicits great hope for molecular-based therapies in many fields, including mast cell-associated disease.

## Potential Targets for New Inhibitors

Although several mast cell mediators and receptors are targeted by existing therapies, others warrant consideration. Tryptase is a mast cell protease that is expressed by all mast cells and contributes to inflammation in atopy and several autoimmune diseases by causing smooth muscle contraction and fibrosis (205, 206). Several beta-tryptase inhibitors have entered clinical trials, with APC 366 moving as far as phase 2a for asthma. However, issues with target selectivity, formulation, and reproductive toxicity have thus far prevented these inhibitors from gaining FDA approval (256). There is potential for antibodies targeting tryptase to be used for mastocytosis and atopic diseases (257). Chymase is another pro-inflammatory protease made in abundance by mast cells with the potential to be targeted by chemical inhibitors or antibodies (258). Several small molecule inhibitors such as ONO-WH-236 have been developed to selectively inhibit chymase, but none of these drugs have been clinically tested in patients with mast cell-associated diseases (205, 259).

As stated above, IL-33 activates mast cells (106, 107, 260) and is elevated in patients with asthma and AD (106, 107, 261, 262). Since it also activates Th2 cells, targeting IL-33 or its receptor ST2 could be effective. An anti-ST2 human monoclonal antibody, MSTT1041A, is in phase 2 of clinical trials to treat severe asthma (http://Clinicaltrials.gov, NCT02918019). In addition, an anti-IL-33 monoclonal antibody is in a phase 2 clinical trial for peanut allergy (http://Clinicaltrials.gov, NCT02920021) and also in phase 2 trials for AD (EU Clinical Trials Register number 2016-002539-14). With varied mechanistic targets for mast cell activation and different classes of therapeutics currently being studied, there is reason to be hopeful for progress in this area.

# CONCLUSION

By demonstrating that mast cells produce a Th2-type profile of cytokines, Bill Paul's group allowed those of us fortunate to work in this field to expand our horizons and our definition of what the mast cell is. The current view is of a long-lived innate immune cell with considerable plasticity that responds to its microenvironment through a range of surface receptors, allowing the mast cell to greatly alter the course of immunity. It is therefore an ideal target for therapeutic intervention and a lineage that still yields novel insights, more than a century after its discovery.

# AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript.

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MINI REVIEW
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# Molecular Regulation of Histamine Synthesis

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Histamine is a critical mediator of IgE/mast cell-mediated anaphylaxis, a neurotransmitter and a regulator of gastric acid secretion. Histamine is a monoamine synthesized from the amino acid histidine through a reaction catalyzed by the enzyme histidine decarboxylase (HDC), which removes carboxyl group from histidine. Despite the importance of histamine, transcriptional regulation of *HDC* gene expression in mammals is still poorly understood. In this review, we focus on discussing advances in the understanding of molecular regulation of mammalian histamine synthesis.

**Keywords: histamine, histidine decarboxylase, enhancers, promoter, gene regulation**

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## INTRODUCTION

Bill Paul's impact on immunology is broad and enormous. Like many of his former trainees, I had the good fortune to learn from him. Bill's mentorship has nurtured my lifelong interest in studying type 2 immune responses that cause allergic diseases and protect against parasitic infections. In the early years of my laboratory, we had investigated how naïve $CD4^+$ T cells commit into T helper type 1 cells by silencing the potential to transcribe the *Il4* gene (1–3). More recently, we extended our efforts to understand how a bi-potential basophil and mast cell progenitor acquires the capacity to transcribe a set of basophil-specific or mast cell-specific genes while simultaneously repressing transcription of a gene set that is specific for the other cell type (4). With a newly gained understanding of a network of transcription factors and their targeted enhancers (5), our laboratory has chosen to investigate the *Hdc* gene (encode histidine decarboxylase, a rate-limiting enzyme for histamine synthesis) in greater detail.

Anaphylaxis is a serious allergic reaction that is rapid in onset and can be life threatening. The clinic manifestations include symptoms that involve the skin, gastrointestinal track, respiratory system, and cardiovascular system (6). Anaphylaxis can be caused by allergy to foods, insect venoms, medications, and other agents (6). The incidence of food-induced anaphylaxis has risen dramatically in developed countries during the past several decades (7–9). The cost of treating food allergy is estimated at about 25 billion dollars annually in the US alone (10).

Histamine plays an essential role in IgE-medicated anaphylaxis, the most common type of anaphylaxis (11–14). Histamine was first purified from ergot fungi (15) in 1910 and from human tissues (16) in 1927. Histamine has pleiotropic effects on skin and the cardiovascular, respiratory, digestive, central nervous, and immune systems (17). It is a profound vasodilator that increases blood vessel permeability, allowing blood leukocytes to enter tissues to promote inflammatory responses. Relatively large quantities of histamine can cause a rapid decrease in body temperature due to massive leakage of blood plasma into the extravascular space. Rapid release of large amounts of histamine leads to anaphylaxis (12, 14). Histamine belongs to a family of biogenic amines that

includes neurotransmitters, such as serotonin and dopamine, and hormones, such as epinephrine. Biogenic amines that contain one or more amine groups are formed mainly by decarboxylation of amino acids. Histamine is a monoamine synthesized from the amino acid histidine through a reaction catalyzed by the enzyme histidine decarboxylase (HDC), which removes carboxyl group from histidine (**Figure 1**). Although histamine can be synthesized by bacteria found in contaminated food (18) and in the gut of asthma patients (17, 19), in this review, we focus on discussing advances in the understanding of molecular regulation of mammalian histamine synthesis.

## HISTAMINE-PRODUCING CELLS IN MAMMALS AND STIMULI THAT TRIGGER HISTAMINE RELEASE

Histamine is synthesized primarily by mast cells, basophils, histaminergic neurons in the basal ganglia of the brain and enterochromaffin-like cells (ECL) in the stomach. These cells produce large amounts of histamine and are thought to be the major histamine-producing cells (**Figure 2**). They continuously synthesize histamine, which is then linked to the carboxyl group of heparin and stored in intracellular granules until the cells receive the appropriate activating stimulus. Upon external stimulation, these cells degranulate, releasing the stored histamine. Stimuli that trigger histamine release by these major histamine-producing cells have been reviewed extensively (20–25). Antigen crosslinking of antigen-specific IgE bound to the high-affinity IgE receptor, FcεRI, on the mast cell and basophil surface is the most robust stimulus that triggers histamine release by these cells (20–23). Substance P and allergy-inducing drugs that bind to G-protein-coupled receptors can also trigger basophils and mast cells to release histamine *via* different signaling pathway (23, 26). In addition, complement components, such as the C3a and C5a "anaphylatoxins," have also been shown to induce histamine release by mast cells (27). Many cytokines, including IL-3, IL-18, IL-33, GM-CSF, and SCF, promote histamine synthesis (28–30). In general, cytokines alone do not induce histamine release although it remains controversial whether IL-33 can have this effect. Some reports describe that IL-33 stimulates histamine release (31, 32), while other reports dispute this (33, 34). It is suggested that IL-33 alone does not induce histamine release by basophils, but enhances histamine release in response to IgE/FcεRI crosslinking (35).

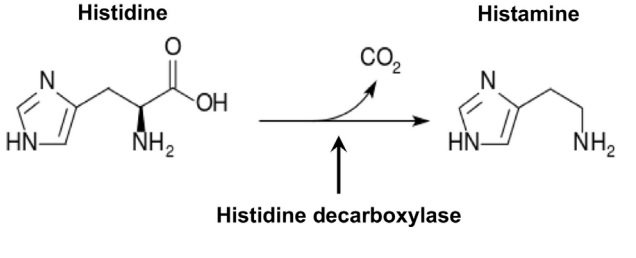


**FIGURE 1** | Histamine synthesis.

Additional histamine-producing cells have also been identified, including T cells (36), dendritic cells (37), macrophages (38, 39), and epithelial cells (40, 41) (**Figure 2**). In contrast to mast cells and basophils, these cells produce relative small quantities of histamine and do not store it in their cytoplasm (42). The small amounts of histamine that they produced are released without external stimulation (42). The biological significance of the small amounts of histamine produced by these minor histamine-producing cells remains unclear. Cell type-specific deletion of the *Hdc* gene, which encodes HDC, an enzyme essential for histamine synthesis, would shed light on the role of histamine synthesis and secretion by the minor histamine-producing cells.

## HDC AND HISTAMINE SYNTHESIS IN MAMMALS

After several groups purified mammalian HDC protein from fetal rat liver and mouse mastocytoma P-815 cells (43–45), a cDNA that encodes this protein was subsequently cloned (46, 47). The *Hdc* gene encodes HDC protein, which has a molecular mass of 74 kDa and is a proenzyme with little or no enzyme activity. Once the proenzyme is cleaved at the site near its c-terminus, presumably by Caspase-9, it yields a 53 kDa N-terminal and a 20 kDa C-terminal subunit. The 20 kDa C-terminal subunit is believed to possess inhibitory activity (48). The 53 kDa N-terminal subunit forms a homodimer that is an active decarboxylase (48, 49). HDC is the primary enzyme that catalyzes histamine synthesis. Mice deficient in the *Hdc* gene fail to synthesize histamine and have reduced or absent IgE-mediated anaphylactic responses (50–53). Several potent HDC inhibitors have been identified, including the histidine derivatives α-fluoromethyl histidine, histidine methyl ester, and pirodoxal histidine methyl ester (54–56). However, these HDC inhibitors have not been further developed for clinical use.

## *HDC* GENE EXPRESSION AND HISTAMINE SYNTHESIS IN BASOPHILS AND MAST CELLS

*Hdc* gene expression and histamine synthesis are regulated both positively and negatively by a range of factors. Notably, crosslinking of FcεRI by antigen binding to FcεRI-associated IgE increases mast cell *Hdc* mRNA expression and histamine synthesis (57, 58). These mast cell activation-induced increases in *Hdc* mRNA expression and histamine synthesis are also induced by phorbol 12-myristate 13-acetate (59). *Hdc* mRNA expression and histamine synthesis also increase as immature mast cells undergo maturation. Bone marrow-derived mast cells (BMMCs) appear immature because they contain relatively little histamine and express relatively low levels of FcεRI (60). These immature mast cells develop into mature mast cells with higher amounts of histamine *in vivo* if they are adoptively transferred into the peritoneal cavity (61). However, it is not clear if *in vivo* exposure to IgE promotes maturation and increases *Hdc* mRNA expression.

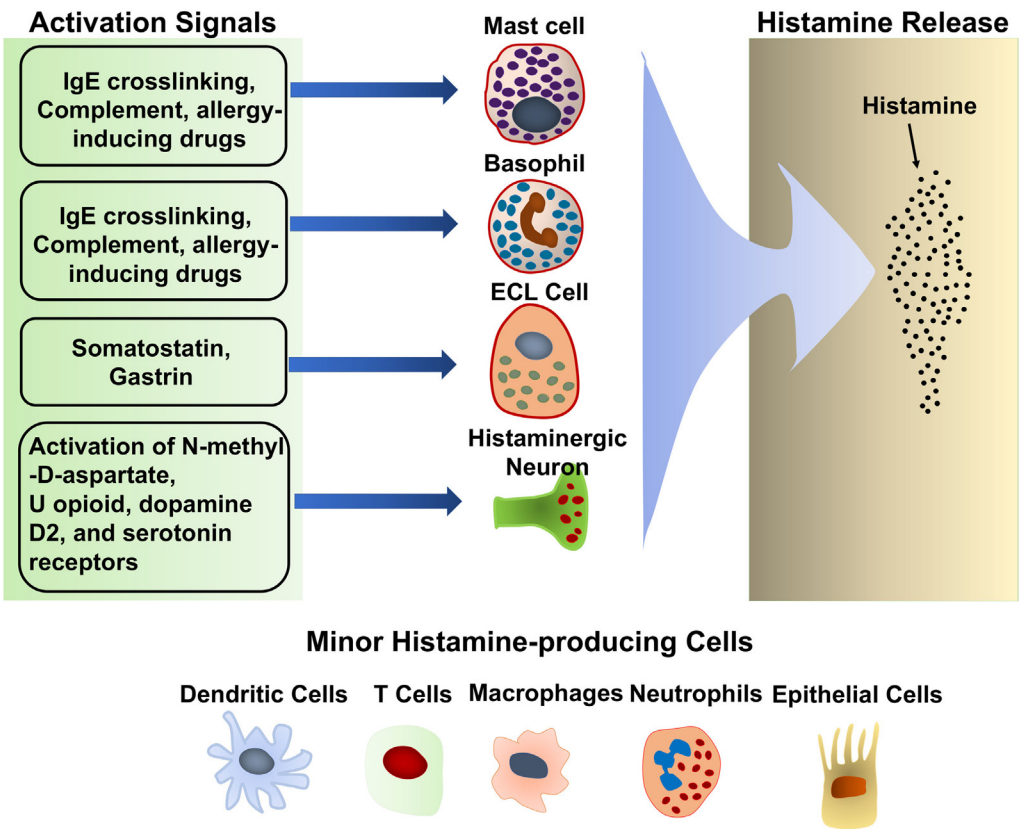


**FIGURE 2** | Histamine-producing cells and stimuli that trigger histamine release.

In this regard, we demonstrated that chlorotoxin, which induces mast cell maturation (62), strongly upregulates *Hdc* gene expression in BMMCs within few hours after the treatment (5). The mechanism by which chlorotoxin enhances *Hdc* gene transcription remains to be determined. It is conceivable that chlorotoxin activates mast cells by binding to an acidic glycosphingolipid, ganglioside G, that has been shown to be expressed on the mast cell surface (62). Chlorotoxin-triggered signals in mast cells then activate transcription factors that directly and rapidly promote *Hdc* gene transcription. It is unknown whether bacteria in the gut of allergic patients can promote *Hdc* mRNA and histamine synthesis by producing substances similar to chlorotoxin.

In line with the notion that factors promoting mast cell maturation also enhance histamine synthesis, cytokines that promote basophil and mast cell maturation, such as IL-3, IL-18, IL-33, GM-CSF, and SCF, have also been reported to increase HDC activity (28–30, 63). It is unclear whether these cytokines regulate *Hdc* gene transcription by increasing the expression of the genes that encode *Hdc* gene-activating transcription factors or by activating already expressed transcription factors to induce transcription of the *Hdc* gene. Other substances, including chemokines, neuropeptide substance P, and IL-1α have also been reported to induce *Hdc* mRNA and histamine synthesis (64, 65).

By contrast, mitochondrial uncoupling protein 2, a mitochondrial transporter protein that transfers anions from the inner to the outer mitochondrial membrane and protons from the outer to the inner mitochondrial membrane, inhibits *Hdc* mRNA expression and histamine synthesis, possibly by suppressing the production of reactive oxygen species (66). Substances found in fruits and vegetables, such as quercetin (67), and in green tea, such as epigallocatechin gallate, also potently inhibit HDC (68). More detailed examination of negative regulators of *Hdc* mRNA expression should promote development of agents that may be able to prevent and treat food allergy and other histamine-mediated allergic inflammatory disorders.

The human *HDC* gene is located in the 15q21.2 region of chromosome 15. It contains 12 exons (69) (**Figure 3**). Eight predicted isoforms can be generated by alternative splicing and two actual isoforms have been described (70). *HDC* mRNA is expressed broadly in many organs, with the highest expression levels found in the gallbladder, stomach, and lung (71). Because the RNA-seq data for normal tissues in the Human Protein Atlas were obtained from intact tissues, it is not clear whether the human *HDC* gene is expressed predominantly in known histamine-producing cells, such as mast cells and ECL in high *HDC*-expressing tissues, or predominantly in other cell types in those tissues. In contrast to

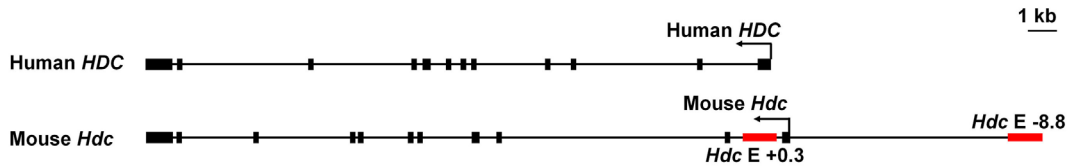


**FIGURE 3** | Genomic structures of the human and mouse histidine decarboxylase (*HDC*) gene. Red bars indicate the enhancers we described.

the human *Hdc* gene, the mouse *HDC* gene is located in chromosome 2 (72). It resembles the human gene in that it contains 12 exons, is expressed broadly in many tissues with the highest expression levels in lung, ovary, and subcutaneous fat pads (72, 73), and is 86% homologous with the human gene (https://www.ncbi.nlm.nih.gov/homologene/20490); however, there are only three predicted isoforms and no isoform, other than the classical one, have been found for murine *Hdc* (72).

There is still limited knowledge of how *Hdc* gene expression is regulated transcriptionally. Most previous work has concentrated on the promoter region of this gene. Deletion analysis of *Hdc* promoter-driven luciferase reporter gene transcription demonstrated that the transcription factor SP1 binds to a GC box (GGGGCGGGG) found in both the human and mouse *Hdc* gene promoters (72, 74). Several promoter elements have been reported to negatively regulate *Hdc* gene transcription. For example, the transcription factors YY1 and KLF4 have been shown to negatively regulate the *Hdc* gene by suppressing SP1 in a gastric cancer cell line (75, 76).

By contrast, *Hdc* gene expression is positively regulated by the transcription factor GATA binding protein 2 (GATA2), a member of the GATA family of transcription factors. GATA2 is critical for survival and proliferation of hematopoietic stem cells (77, 78), granulocyte-monocyte progenitor differentiation (79), and basophil and mast cell differentiation (80, 81) and is required for connective tissue mast cell development (5). By contrast, basophil development is not affected in connective tissue-specific *Gata2*-deficient mice (5). We have also found that mucosal and connective tissue-specific *Gata2*-deficient mice fail to develop both mucosal and connective tissue mast cells, indicating that GATA2 is required for both mucosal and connective tissue mast cell development (Li et al., unpublished data). To distinguish the role of GATA2 in regulating the *Hdc* gene from its role in mast cell development, we used an inducible gene deletion method to delete the *Gata2* gene from mast cells after they had fully differentiated. In this inducible gene deletion model, the enzyme Cre is fused to the estrogen receptor (ER) and the ER-Cre fusion product is induced to enter the cell nucleus to cleave a floxed gene of interest by the ER ligand 4-hydroxytamoxifen (82). Using this method, we demonstrated that GATA2 plays a critical role in regulating *Hdc* gene expression in even fully differentiated mast cells. However, in contrast to its role in mast cell development, GATA2 is not needed for survival of fully differentiated mast cells (83).

More recently, our group has used active histone mark ChIP and reporter gene transcription assays to identify and characterize two *Hdc* enhancers in mast cells. Epigenomic studies demonstrate that monomethylation of lysine 4 on histone 3 (H3K4me1) marks genes that are poised to be transcribed, whereas acetylation of lysine 27 on histone 3 (H3K27ac) identifies genes that are actively being transcribed. The combined presence of H3K4me1 and H3K27ac modifications predicts enhancer activity (84–88). Our H3K4me1 and H3K27ac ChIP-seq analysis of BMMCs identified two putative *Hdc* enhancers located −8.8 kb upstream and +0.3 kb downstream from the transcription start site of the *Hdc* gene (**Figure 3**). We demonstrated that the −8.8 kb *Hdc* enhancer, but not the +0.3 kb *Hdc* enhancer, increases minimal *Hdc* promoter activity in a luciferase reporter gene transcription assay. The transcription factor MITF binds to the −8.8 *Hdc* enhancer and drives its enhancer activity. Indeed, MITF overexpression largely restores *Hdc* gene expression in *Gata2*-deficient mast cells. Our study also suggests that GATA2 induces MITF and that these two transcription factors together direct full *Hdc* gene transcription in mast cells in a feed-forward manner. However, it is not certain that the −8.8 kb *Hdc* enhancer is fully responsible for positive regulation of the *Hdc* gene, because *in vivo* importance of the +0.3 kb *Hdc* enhancer in *Hdc* gene transcription cannot be ruled out by the luciferase reporter gene transcription assay alone (5).

Despite remarkable progress in genome-wide annotation of potential enhancers, functional validation of annotated enhancers remains an unmet challenge. Transgenic mice, reporter gene assay, and CRISPR/Cas9 genome editing have been used to validate the biological functions of enhancers identified by histone marks. Each of these methods has its strengths and weaknesses (89, 90). The reporter gene assay has been widely used to assess enhancer activity. It is simple, rapid, and efficient at assessing promoter and enhancer activity in transiently or stably transfected cell lines. The limitation of the transient reporter gene assay is that it does not measure promoter and enhancer activity in the context of chromatin. Despite this disadvantage, this reductionist approach is useful for assessing binding of transcription factors to *cis* regulatory elements in accessible regions. It has been reported that ~60% of annotated enhancers show enhancer activity by the luciferase reporter gene assay (86, 91–94). The *in vivo* function of the −8.8 *Hdc* enhancer requires further investigation.

## HISTAMINE SYNTHESIS IN THE CENTRAL NERVOUS SYSTEM AND THE STOMACH

In addition to its activity as a vasoactive mediation, histamine is a neurotransmitter and a regulator of gastric acid secretion. *HDC* mRNA is expressed in the brain exclusively in the basal ganglia (95). Specific ablation of histaminergic neurons leads to repetitive movements (96), that resemble the signs of Tourette syndrome (97). Consistent with this, a nonsense mutation at the human

*HDC* gene (W317X) has been identified in a family of patients with this syndrome (97, 98) and mice completely deficient in *Hdc* gene transcription develop a Tourette-like syndrome (97, 99). However, the mechanisms involved in *Hdc* gene regulation in the basal ganglia are currently unknown. In the stomach, histamine is synthesized in ECL and is released from these cells upon gastrin and acetylcholine stimulation. The released histamine then stimulates parietal cells to secrete stomach acid (25, 100). Mice deficient in the *Hdc* gene fail to fully acidify their gastric contents (100), which can lead to indigestion, diarrhea, constipation, or rectal itching (101). Clinically, histamine 2 ($H_2$) receptor antagonists, such as ranitidine, are currently used to ameliorate stomach hyperacidity and peptic ulcer disease by blocking this receptor on the hydrochloric acid-producing parietal cells in the stomach (102). At present, it is not known how the *Hdc* gene is regulated in ECL. It is most likely that different transcription factors are used to regulate the *Hdc* gene in basal ganglia and ECL.

## CONCLUDING REMARKS

Histidine decarboxylase is the rate-limiting enzyme for histamine synthesis. Understanding transcriptional regulation of the *Hdc* gene will advance our knowledge about how this gene detects extracellular stimuli and increases its transcription, leading to histamine synthesis, replenishment, and accumulation that exacerbate allergic inflammation and anaphylaxis. Fine mapping of critical transcription factors and their authentic binding sites within the *Hdc* promoter and enhancers should promote identification of regulatory variants that influence allergy susceptibility and severity. Today, Bill Paul's teaching and his large body of work on IL-4 continues to inspire our fascination with type 2 immunity.

## AUTHOR CONTRIBUTIONS

All authors contributed to the literature review and writing the paper.

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REVIEW
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# Multilayer Control of B Cell Activation by the B Cell Antigen Receptor: Following Themes Initiated With Bill Paul

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This article describes the work I did in Bill Paul's lab as a postdoctoral fellow between 1979 and 1983, and to a lesser extent puts that work in the context of other work on B cell activation and antibody responses that was going on in Bill's lab at that time and shortly beforehand, including the discovery of interleukin 4. In addition, this work describes the subsequent and continuing work in my own lab following-up on themes I began during my time working directly with Bill. A particular emphasis was on understanding the biochemical mechanisms of signaling by the B cell antigen receptor (BCR) to the interior of the B cell. Some of the studies from my lab related to the regulation of BCR signaling by Lyn are described in relationship to the lymphocyte tuning hypothesis put forth by Grossman and Paul in 1992 and subsequently.

**Keywords: activation, autoimmunity, B cell, B cell antigen receptor, signaling, tolerance, tuning**

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## STUDIES OF B CELL ACTIVATION WITH BILL PAUL (1979–1983)

I joined Bill Paul's research group at NIH in late 1979 as a postdoctoral fellow soon after Bill had focused much of his attention on understanding B cell activation by antigen. I was interested in the biochemical mechanisms by which receptors signaled to the interior of the cell that they had bound their ligand. At this time, before the discovery of the TCR (which was very close on the horizon; Mark Davis joined us a year later), this problem was more readily approachable in B cells than T cells, as anti-Ig reagents were an accepted surrogate for antigen, and there was no equivalent approach in T cells.

Previous to this time, Bill and colleagues had studied how lymphocytes responded to antigen in B cells and T cells and had concluded that membrane Ig on the surface of B cells was a signaling receptor (1), as opposed to serving primarily a binding/focusing function, as proposed by some other investigators. For example, they and others had found that anti-IgM crosslinking antibodies induced vigorous proliferation of mouse B cells. My initial studies with Bill were designed to characterize in more detail the nature of this proliferation. Maureen Howard, who had recently joined Bill's lab from Australia, used this assay to look for T cell-derived growth factors for B cells (by analogy with IL-2), and she and John Farrar discovered IL-4 by its ability to strongly promote the proliferation of B cells stimulated with a sub-mitogenic concentration of anti-IgM antibodies (2).

The spleen was used as a source of B cells for these experiments, and there was evident heterogeneity in the size of the cells, suggesting that perhaps some of the cells were in the process of activation at the time of isolation. It is now known that splenic marginal zone B cells and B1 B cells have an enlarged pre-activation-like phenotype, in contrast to follicular B cells, which are small, resting lymphocytes. To reduce this heterogeneity, I used Percoll density gradients to isolate a more homogeneous small

lymphocyte population of splenic B cells. These cells, comprising 60–70% of splenic B cells, fit the cell biological definition of resting or quiescent cells, as they needed at least 30 h of stimulation before entering S phase and completed the first round of cell division in a synchronous fashion (3, 4). When stimulated with anti-IgM, all of these small, resting B cells exhibited a prolonged period of cell enlargement, corresponding to exit from a quiescent phase ($G_0$) and progress through the $G_1$ phase of the cell cycle in a process that required continuous stimulation (5). At any time during the first 24 h, removal of anti-IgM caused the cells to stop their enlargement, indicating that progression through early $G_1$ phase was dependent on continued B cell antigen receptor (BCR) stimulation (5). This result was somewhat surprising since anti-IgM is very effective at capping membrane IgM molecules and causing their internalization and degradation. However, newly synthesized membrane IgM molecules are present on the cell surface, albeit at low levels, and these studies indicated that their engagement and signaling was required for B cell activation to proceed. After 24 h, progression through S phase, which occurred with about 50% of the stimulated B cells, was now independent of BCR stimulation, consistent with B cells following the cell cycle rules observed in various other mammalian cell types in culture.

Previous work in Bill's lab had found that anti-IgM failed to induce proliferation of splenic B cells isolated from CBA/N mice or from $F_1$ male mice with CBA/N mothers (6), which were subsequently shown to have a loss-of-function mutation in the gene encoding Btk (7), which is located on the X chromosome. Btk is now known to be an important signaling component of the BCR. The mutant locus in CBA/N mice at the time was called *xid*, for X-linked immunodeficiency locus, as these mice had defective antibody responses to polysaccharide antigens. These antigens would induce antibody responses in T cell-deficient mice (nude mice), as would some other antigens that induced responses in *xid* mice. Thus, T cell-deficient mice and *xid* mice were used to characterize antigens into three functionally distinct groupings: T cell-dependent antigens, T-independent type I antigens (those that worked in *xid* mice), and T-independent type 2 antigens (those that did not induce antibody responses in *xid* mice). Based on lack of responsiveness in *xid* mice, anti-IgM most resembled polysaccharide antigens (TI-2 antigens), which made sense in that polysaccharides were thought to be able to effectively cross-link many BCR molecules on the surface of B cells (8) and hence induce strong signaling reactions to stimulate the B cell, a point that was experimentally verified several years later when BCR signaling reactions were identified (9). This analogy only went so far, however, as anti-IgM-stimulated B cells failed to differentiate into antibody-secreting cells *in vitro*, suggesting that additional signals beyond BCR signaling were needed. Along with Maureen Howard and Bill's pioneering discovery of IL-4, two other groups discovered IL-5 and IL-6, which had distinct effects on B cells *in vitro*. Treatment of anti-IgM-stimulated B cells with highly purified IL-4$^+$IL-5$^+$IL-6 induced them to terminally differentiate into antibody-secreting cells (10), thereby providing an *in vitro* model mimicking many properties of polysaccharide antigens.

Bill's interest in using *xid* mice as a tool to uncover aspects of B cell activation in this time period contributed importantly to understanding the differential requirements for antibody responses of polysaccharide antigens vs. other types of antigens and several of my fellow postdoctoral fellows in Bill's lab were studying antibody responses to pure polysaccharide antigens (11, 12). Remarkably, the understanding that Bill's lab contributed on this topic would subsequently have relevance to human vaccine design. To make vaccines against several major bacterial pathogens, their cell wall polysaccharides were isolated and used as vaccines. It was subsequently recognized that this type of vaccine was poorly efficacious in very young children (<2 years old), whereas other types of vaccines were effective when used to immunize children several times within the first year of life. Thus, the TI-2 vaccines had limitations that meant that they were unable to prevent some forms of serious disease in young children. The elegant solution was to convert TI-2 antigens to T cell-dependent antigens by attaching an immunogenic protein to them, creating the "conjugate vaccines" (13). Although Bill's own research efforts were not directed toward this particular development, his earlier studies had laid the conceptual groundwork for the development of conjugate vaccines.

While IL-4, IL-5, and IL-6 could all be made by CD4$^+$ T cells, the anti-IgM$^+$IL-4$^+$IL-5$^+$IL-6 model did not seem to fully recapitulate the activity of helper T cells, in part because *xid* B cells could not respond in this system, but made reasonable responses *in vivo* to T cell dependent antigens such as haptenated proteins. At that time, Ron Schwartz's lab, also in the Laboratory of Immunology at NIH, had become highly proficient at propagating CD4 T cells *in vitro* and could generate clonal cell lines with homogeneous specificity. One of Ron's postdoctoral fellows, Jonathan Ashwell, now an investigator at NCI, had such T cell clones, and we decided to join forces to try and study how helper T cells and B cells interact to induce T cell-dependent antibody responses. We were able to observe excellent polyclonal proliferation of small resting splenic B cells when we put them together with some of Jon's clones and added the antigen for that clone. This represented a polyclonal version of earlier experiments published by Singer and colleagues at NIH, who had taken antigen-specific helper T cells, combined them with B cells and achieved *in vitro* activation of the antigen-specific B cells as judged by antibody production. In those studies, to be activated, the B cells had to express the allelic form of class II MHC that was recognized by the helper T cells (14). We thought our system might be able to tease out some aspects of the mechanism by which helper T cells activate B cells, and indeed this was the case, but only after an important issue was resolved first.

Central to these experiments was the issue of whether B cells presented antigen to T cells and if so, what were the functional consequences of that presentation for the two partners in the interaction. Since B cells expressed high levels of class II MHC molecules, it seemed likely that they could present antigen to T cells but did this presentation lead to activation of the T cells or did the recognition of peptide/MHC by the T cell directly send an activation signal to the B cell? With regard to the activation of the T cell, B lymphoma-derived cell lines could present antigen to primary T cells (15), but attempts to demonstrate directly this presentation by primary B cells *in vitro* had often been unsuccessful. We were more focused on the other issue: would the clonal T cells, once activated by adding their antigen, stimulate any

B cell or only the B cell presenting antigen to that T cell? If T cell help for B cells was primarily mediated by the cytokines produced upon T cell recognition of antigen, then perhaps the T cell would activate "bystander" B cells. Alternatively, the recognition of peptide/MHC by the T cell might generate a signal only within the antigen-presenting B cell, for example, transmitted by MHC class II molecules upon their engagement by the T cells' TCR. To address this question, we mixed together with the T cell clones equal numbers of two types of splenic B cells, one expressing the allele of class II MHC molecule that was recognized by the T cell, and the other expressing only non-stimulatory alleles of MHC class II. As the read out for many of these experiments was proliferation of the cell of interest, a common approach was to irradiate the other cells added to the culture, so that they were incapable of incorporating radiolabeled thymidine and hence would not add to the signal. When an unseparated population of spleen cells was irradiated in this way (3,000 R), it retained full activity to activate T cells, so this approach seemed to be valid. For our experiments, we irradiated the T cells and one or the other of the two B cell populations. When we then analyzed proliferation of the two types of B cell, the B cells with the correct MHC class II proliferated when the bystander B cells were irradiated, but the bystander B cells failed to proliferate when the antigen-presenting B cells were irradiated. This turned out to be a misleading answer, as we soon discovered. In discussing this experiment with Bill and Ron Schwartz, Bill was concerned that perhaps the effects seen were due to differential activation of the clonal T cells in the two parallel cultures. While the irradiation procedure seemed to be innocuous, how could we be sure? Bill's rigorous thought proved to be pivotal. Jon and I went back to the bench and devised an experiment to address Bill's objection. Now, we did not irradiate either B cell population, but just mixed them together, incubated them with the T cell clone and added the T cell's antigen. After 24 h incubation, we measured enlargement of the two types of B cells, which we could distinguish by flow cytometry. Now both the antigen-presenting B cells and the bystander B cells became activated to similar extents, indicating that once the T cells were activated, they could activate bystander B cells as well as the B cells that presented antigen to them (16). Of course, the activation of the T cells was MHC restricted, but, at least in this circumstance where there were many T cells present, the means by which T cells activated B cells was not MHC restricted and behaved as expected for cytokines. It was several years later that Randy Noelle at Dartmouth University discovered the molecular mechanism for this activation as being due to CD40L on the surface of the helper T cell (17). T cell recognition of antigen/MHC induces upregulation of CD40L on the T cell, which delivers a critical signal to B cells *via* their CD40. We now know that soluble cytokines such as IL-4 and IL-21 also contribute to the B cell response. The critical nature of CD40L for helper T cell activation of B cells was subsequently verified by the discovery that X-linked hyper-IgM syndrome, in which T cell-dependent antibody responses are highly defective, results from mutations in the gene encoding CD40L (18).

A second outcome of the collaboration between Jon and me was the direct demonstration that primary B cells are very good at presenting antigen to T cells and activating them (19). Whereas macrophages and dendritic cells still presented antigen well following irradiation at 3,000 R, antigen presentation by B cells was very sensitive to irradiation; their antigen presentation function could be maintained by irradiation up to 1,000 R, but above that, their antigen presentation function was abrogated by irradiation-induced apoptosis. Thus, our early experimental design was flawed by a difference in radiosensitivity between small resting B cells and "professional" antigen-presenting cells such as macrophages and dendritic cells. In any case, the direct demonstration of a robust ability of primary B cells to present antigen to T cells and induce their activation, an outcome of this project, was a substantial contribution to immunology at the time, and by challenging Jon and me to improve our experiments, Bill played an essential role in this discovery.

## BIOCHEMICAL BASIS OF BCR SIGNALING (1983–PRESENT)

As I was pursuing the projects described earlier in Bill's lab at NIH, I was interested in the biochemical basis by which BCR engagement by anti-IgM induced its effects on B cells. In those days, knowledge about receptor signaling mechanisms was limited to a handful of receptors. Given the techniques available at the time, investigating the process in small resting splenic B cells seemed to be a large challenge. Mark Davis was accumulating different mouse B lymphoma-derived cell lines to use in his efforts to isolate and characterize genes that were differentially expressed between B cells and T cells. Mark and Steve Hedrick found that among the cDNAs that were expressed only in T cells, one was found to exhibit DNA rearrangement in the genome of T cells and was then found to encode the TCR β chain, which was the initial cloning of a TCR gene (20). One of the B cell lines accumulated by Mark, WEHI-231, stopped growing when incubated with anti-IgM, demonstrating that it had intact BCR signaling and suggesting that it might be representative of immature B cells contacting self-antigen (3, 4). While in Bill's lab, I explored the properties of this cell line and decided that when I started my own lab I would use this cell line to study the mechanism of BCR signaling.

Of course how antigen receptors informed B cells and T cells of their encounter with cognate antigen was a fundamental problem and of great interest to many immunologists. Roger Tsien had recently synthesized novel calcium sensing fluorescent dyes that could be loaded into cells and used to measure intracellular free calcium which was widely thought to be important for regulating cell responses and indeed, it turned out that both B cells and T cells stimulated *via* the BCR or TCR exhibited a rapid and robust increase in intracellular free calcium (21). At this time, a novel lipid signaling reaction, involving hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) had been recently characterized in a number of different cell types and Gerry Klaus and collaborators then showed that B cells stimulated by the BCR robustly triggered this signaling reaction (22). We confirmed that this was also true of the WEHI-231 cell line and that one of the second messengers released, inositol 1,4,5-trisphosphate could release calcium from intracellular stores present in B cells, as in smooth muscle and other non-lymphoid cell types (23),

indicating that hydrolysis of $PIP_2$ was likely upstream of the rapid rise in intracellular free calcium seen upon treatment of B cells with anti-IgM. $PIP_2$ hydrolysis seemed to be an important aspect of lymphocyte response to antigen as in both B cells and T cells, since antigen stimulation could be mimicked well by incubating them in a calcium ionophore (to raise intracellular free calcium) and a phorbol ester (24), compounds that were unmetabolizable analogs of the other second messenger generated from $PIP_2$ hydrolysis, namely diacylglycerol (DG). At the time, DG and phorbol esters were thought to act *via* a small family of protein kinases called protein kinase Cs. Several years later, it was learned that there are additional signaling reactions downstream of DG or phorbol esters, including activation of the Ras GTPases *via* RasGRP. DG activation of RasGRP is a critical pathway in lymphocytes leading to activation of mitogen-activated protein kinases (25, 26).

As appreciation of the likely important role of $PIP_2$ hydrolysis as a mediator of BCR and TCR signaling grew, the problem remained, how did these receptors activate this signaling reaction? The answer emerged from investigators studying how cells became malignant. Cancer researchers Tony Hunter, Mike Bishop, and others, had implicated a new form of protein phosphorylation, tyrosine phosphorylation, as central to growth control in multiple situations, including treatment of cells with growth factors and transformation of cells with certain oncogenes. It emerged that the epidermal growth factor receptor's intracellular domain was a tyrosine kinase and other tyrosine kinases, such as the viral oncogene v-Src and its normal cellular counterpart (c-Src), were intracellular protein tyrosine kinases associated with the plasma membrane. Mike Gold in my lab used recently developed antibodies against phosphotyrosine to demonstrate that stimulation of the BCR induced very rapid tyrosine phosphorylation of a series of different proteins (27), a result that was also reported independently at about the same type by several other groups (28). In parallel, protein tyrosine phosphorylation emerged as a critical early event triggered by the TCR, with major contributions made by Larry Samelson, Andre Veillette, Joe Bolen, Art Weiss, and others (29).

Soon after the discovery that BCR and TCR stimulation induced tyrosine phosphorylation of multiple proteins, it became evident these phosphorylations represented the key proximal signaling events triggered by antigen engagement (27). For example, Mike Gold and others in my lab set about defining the targets of this phosphorylation (28, 30–32), which made it possible to connect tyrosine phosphorylation to other signaling events such as $PIP_2$ hydrolysis and calcium elevation.

In addition to identifying some of the signaling molecules that were the targets of this tyrosine phosphorylation, we set about identifying the tyrosine kinases that were activated and understanding how they associated with the BCR. We found that among the earliest phosphorylated proteins were the membrane Ig associated proteins Igα and Igβ (CD79a and CD79b) (33), and moreover, only the Igα and Igβ of engaged BCR complexes became tyrosine phosphorylated, whereas these subunits of unbound BCR complexes did not become tyrosine phosphorylated. These tyrosines are present in a sequence motif also found in TCR signaling chains (CD3 γ, δ, and γ and ζ chain) and in other immune receptors with similar signaling mechanisms, such as activating Fc receptors, as first identified by Reth (34) and now referred to as the immunoreceptor tyrosine-based activation motif (ITAM). Art Weiss's group discovered a tyrosine kinase, ZAP70, that binds to TCR ζ phosphorylated ITAMs (35) and is required for their signaling. B cells and most other hematopoietic cell types do not express ZAP70, but do express a very similar protein tyrosine kinase, Syk, and a number of groups showed that it plays an analogous role in BCR signaling (36–39).

These observations left open the question of what tyrosine kinase phosphorylates the ITAM tyrosines, and Src-family tyrosine kinases were implicated as performing this role in T cells (29). B cells primarily express three Src-family tyrosine kinases, Lyn, Fyn, and Blk. To address the role of Lyn, my colleague Cliff Lowell and others generated mice with an inactivated Lyn gene (40–42), and Tomohiro Kurosaki inactivated the Lyn gene in a chicken B cell line, DT-40 (37). In the DT-40 cells, Lyn is apparently the predominant Src-family tyrosine kinase, and the deletion of either Syk or Lyn is sufficient to largely cripple BCR signaling, consistent with the concept that these two types of tyrosine kinases must work in concert to mediate BCR signaling. Genetic analysis of B cells from mice deficient in Lyn, Fyn, or Blk demonstrated that these three Src-family tyrosine kinases are redundant for BCR crosslinking-induced phosphorylation of BCR ITAMs and only deletion of all three results in a block in B cell development at the pre-BCR checkpoint (43). Consistent with a redundant function of Lyn, Fyn, and Blk for initiation of BCR signaling, we found that B cells from Lyn-deficient mice had a slower initiation of BCR-induced calcium elevation and tyrosine phosphorylation of cellular proteins, compared with wild type B cells (40, 44).

## THE TUNABLE LYMPHOCYTE ACTIVATION THRESHOLD IN B CELLS: REGULATION OF mIgM EXPRESSION AND B CELL ANERGY (1992–PRESENT)

In 1992, Zvi Grossman and Bill Paul proposed a hypothesis that the activation threshold for T cell activation was not a constant with regard to the amount of antigen sensed, but rather was "tuned" by the subthreshold antigen receptor signals that the T cell had received in the recent past (45). This tuning was viewed as transient and an adaptive response to the presence of self-antigens that induced frequent but low intensity antigen receptor signals. Numerous subsequent studies described the applicability of this concept to developing and mature T cells in various situations, and moreover indicated that B cells and NK cells also adapt themselves to their degree of sub-activation stimulation through the BCR and NK cell receptors, respectively (46). In this section, I describe how the Grossman and Paul tuning hypothesis may relate to the regulation of BCR signaling, which has been studied by my lab and by many other labs. In this regard, it should be noted that Grossman and Paul distinguished lymphocyte tuning, which is transient, from developmental or differentiative changes in antigen receptor signaling, such as durable differences that are characteristic of double positive thymocytes vs. naïve T cells or of naïve T cells vs. memory T cells.

A remarkable heterogeneity of mature resting B cells is seen in the level of expression of mIgM, which is in contrast to mIgD, which is expressed by all mature resting B cells at a comparable level. Membrane IgD is not expressed in the most immature B cells in bone marrow and spleen, referred to transitional 1 or T1 B cells, whereas it is highly expressed in mature B cells. Chris Goodnow and colleagues found that mIgM was strongly downregulated in the MD4 Ig transgenic B cells when they came from a mouse expressing the corresponding antigen, lysozyme, as a self-antigen (47, 48). In addition, these B cells were profoundly unresponsive to stimulation *in vitro* or *in vivo*, a condition referred to as anergy. Subsequent studies have found that downregulation of mIgM is a function of the degree of self-antigen recognition for a particular B cell specificity and therefore the majority of mature B cells in the periphery exhibit some degree of self-reactivity (49). Studies of BCR signaling indicate that the downregulation of mIgM also decreases signaling observed when mIgM is crosslinked with anti-IgM antibody or when specific antigen is used in a low valency form, e.g., a form that would not induce strong crosslinking of mIgM molecules (47, 48). Thus, mIgM downregulation is a tuning response of B cells to adjust to their level of self-reactivity. Interesting in this regard, low valency antigens induce robust signaling from mIgM, whereas they are poor stimulators of mIgD on the same cells (50). By contrast, mIgD is able to signal vigorously if extensively crosslinked. Thus, the tuning of the antigen responsiveness of B cells that is observed is primarily applicable to self-antigens that are poorly able to crosslink BCRs. This implies that a foreign antigen present in a higher valency form (as would often be the case on a virus particle or a microbial cell surface) would still be able to stimulate vigorously those B cells that have tuned their responsiveness due to some self-reactivity.

While the Grossman and Paul concept of lymphocyte tuning and the concept of B cell anergy both address the result of B cell self-reactivity, the former concept emphasizes the potential of these cells to participate in immune responses, whereas the latter concept focuses attention of the role of tuning in restraining their activation and the maintenance of immune tolerance to self. Despite this difference in outlook, the phenomenon of B cell anergy is best understood by considering it in light of the continuum of mIgM downregulation seen in the normal population of follicular B cells and the concept of lymphocyte tuning. That is to say, B cell anergy should be considered as the property of those B cells with greater degrees of tuning and moreover, the functional defects in anergic B cells also likely represent a continuum from a deeper anergy, as seen in the MD4 anti-lysozyme Ig transgenic mice and as seen in those anti-DNA reactive B cells in which the IgH transgenic 3H9 heavy chain is paired with λ1 light chains (51), to a milder anergy, as seen in B cells from the Ars/A1 transgenic mouse. Milder forms of B cell anergy appear to be very rapidly reversible, probably reflecting primarily changes in localization and activity of signaling regulators within the cell (52), whereas more deeply anergic B cells have a more slowly reversible tuning of their responsiveness, which may in part reflect transcriptional changes in the levels of signaling regulators (47).

## REGULATION OF BCR SIGNALING BY THE INTRACELLULAR PROTEIN TYROSINE KINASE Lyn (1995–PRESENT)

Our studies with B cells from Lyn-deficient mice also revealed that Lyn has a second function in B cells in addition to its phosphorylation of BCR ITAMs. While BCR signaling exhibited a short delay in reaching its peak, at later times BCR signaling was elevated in $Lyn^{-/-}$ B cells compared with wild type B cells. This enhanced BCR signaling results from the loss of Lyn's unique ability to attenuate BCR signaling by phosphorylation of tyrosines in the cytoplasmic domains of inhibitory receptors, including FcγRIIb and CD22 (44, 53). Phosphorylation of single tyrosines within conserved sequences, called immunoreceptor tyrosine-based inhibitory motifs leads to recruitment to the plasma membrane of inhibitory phosphatases, both SHP-1, which is a protein tyrosine phosphatase that counters BCR signaling at early stages (54), and SHIP-1, a lipid phosphatase that removes the signaling lipid phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$), and thereby attenuates a critical branch of the BCR signaling pathway (55). *In vitro* BCR signaling in response to anti-IgM reagents that do not bind to FcγRIIb are similarly enhanced in $Lyn^{-/-}$ follicular B cells and in $CD22^{-/-}$ follicular B cells (56), indicating that phosphorylated CD22 provides a tonic inhibition of BCR signaling and that *in vitro*, it is the main inhibitory receptor in B cells downstream of Lyn.

Lyn-deficient mice develop a severe lupus-like autoimmunity, characterized by production of anti-nuclear antibodies (ANAs) starting at about 3 months of age and die of glomerulonephritis after a little more than 1 year of age (53). Deletion of *Lyn* selectively in B cells using Mb1-Cre to induce deletion from a floxed allele of *Lyn* is sufficient to induce ANAs and glomerulonephritis (57), and similar phenotypes are found in $CD22^{-/-}$ mice (58), $FcγRIIb^{-/-}$ mice (59), and mice with a B cell-specific deletion of the gene encoding SHP-1 (54). Lyn is also expressed in dendritic cells, macrophages, and neutrophils and also enables inhibitory receptor function in these cell types. Deletion of *Lyn* selectively in dendritic cells is also sufficient to induce a lupus-like autoimmunity including production of ANAs, but with greater barrier inflammation than is seen in $Lyn^{-/-}$ mice (60). Thus, the lupus-like autoimmunity seen in $Lyn^{-/-}$ mice is driven both by Lyn-deficient B cells and by Lyn-deficient dendritic cells. The ANAs produced in $Lyn^{-/-}$ mice are T cell-dependent and are dependent upon MyD88-dependent signaling in B cells (61). MyD88 is a signaling adaptor that is essential for signaling by most toll-like receptors, including TLR7 and TLR9.

Thus, defects in Lyn, the inhibitory receptors that it phosphorylates in B cells, or the inhibitory phosphatases that become recruited by those inhibitory receptors upon their phosphorylation, all predispose mice to lupus-like autoimmunity, suggesting a critical role for Lyn-mediated attenuation of BCR signaling in preventing autoantibody production to nuclear components. Production of these particular autoantibodies depends on MyD88 signaling in the B cells, both in $Lyn^{-/-}$ mice and in other mouse models of lupus that have been examined (62, 63). It appears that the nucleic acid present in apoptotic debris can promote

activation of DNA or ribonucleoprotein-specific B cells *via* a synergy between exaggerated BCR signaling (resulting from loss of Lyn-mediated inhibitory pathways) and TLR7 or TLR9 signaling in the B cell. In support of this concept, it is clear that TLR7 or TLR9 can greatly enhance B cell responses to virus-like particles that contain ligands for TLR7 or TLR9 (64), or to hapten-carrier conjugates with attached TLR9 ligands (65) and this requires cell-intrinsic TLR signaling in the responding B cell.

Lyn-mediated inhibitory pathways may be one mechanism of tuning of B cells, as described by the Grossman and Paul lymphocyte tuning hypothesis. The downregulation of mIgM cell surface expression is likely to be a major mechanism of B cell tuning, and this function appears to occur normally in Lyn-deficient B cells. Nonetheless, Lyn-mediated inhibitory signaling may be a second important mechanism of tuning of B cells. Although the molecular mechanisms of tuning of T cells are not entirely defined, various studies suggest that upregulation of the inhibitory receptor CD5 is one mechanism of tuning of T cells and recruitment of SHP-1 to active TCRs represents another mechanism (46). Thus, an involvement in B cells of Lyn, CD22 and SHP-1 in tuning would be consistent to what is currently known about the signaling mechanisms of tuning of T cells. Moreover, various studies indicate that anergic B cells exhibit striking attenuation of BCR signaling (66) and moreover this attenuation has the hallmarks of tuning, as it is induced by chronic contact with self-antigen and is rapidly reversible if the self-antigen is removed. Lyn inhibitory pathways are likely to be at least partially responsible for the attenuation of BCR signaling seen in anergic B cells since the maintenance of anergy requires the presence of both SHP-1 and SHIP-1 (52), both of which are largely dependent upon Lyn for their recruitment to inhibitory receptors in the plasma membrane.

## CONCLUDING THOUGHTS

Bill Paul's great contributions to the field of immunology came in many spheres and in many ways (1). In this article, I have discussed Bill's influence on me as I entered the field of immunology and discussed how his theoretical contributions regarding modulation of the strength of antigen receptor signaling has considerable relevance to some of the experimental systems that I have pursued in my own independent laboratory in the years since my time as a postdoctoral fellow in his lab. Clearly, Bill had a long-lasting and positive impact on the field of immunology in many ways, but his roles as a mentor and as a thought leader in developing overriding concepts about how the immune system functions were two of his more important impacts.

## AUTHOR CONTRIBUTIONS

AD wrote the text and is wholly responsible for the opinions expressed.

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REVIEW
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# Unique Action of Interleukin-18 on T Cells and Other Immune Cells

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Interleukin (IL)-18 was originally discovered as a factor that enhances interferon (IFN)-γ production by anti-CD3-stimulated Th1 cells, particularly in association with IL-12. IL-12 is a cytokine that induces development of Th1 cells. IL-18 cannot induce Th1 cell development, but has the capacity to activate established Th1 cells to produce IFN-γ in the presence of IL-12. Thus, IL-18 is regarded as a proinflammatory cytokine that facilitates type 1 responses. However, in the absence of IL-12 but presence of IL-2, IL-18 stimulates natural killer cells, NKT cells, and even established Th1 cells to produce IL-3, IL-9, and IL-13. Thus, IL-18 also facilitates type 2 responses. This unique function of IL-18 contributes to infection-associated allergic diseases. Together with IL-3, IL-18 stimulates mast cells and basophils to produce IL-4, IL-13, and chemical mediators such as histamine. Thus, IL-18 also induces innate-type allergic inflammation. IL-18 belongs to the IL-1 family of cytokines, which share similar molecular structures, receptors structures, and signal transduction pathways. Nevertheless, IL-18 shows a unique function by binding to a specific receptor expressed on distinct types of cells. In this review article, I will focus on the unique features of IL-18 in lymphocytes, basophils, and mast cells, particularly in comparison with IL-33.

**Keywords: interleukin-18, Th1, interferon-γ, interleukin-4, innate-type allergy, interleukin-33, ILC2**

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## PREFACE

I worked with Dr. William E. Paul from 1981 to 1984 in Laboratory of Immunology (LI), National Institutes of Allergy and Infectious Diseases, National Institutes of Health. As he was a laboratory chief at LI, I assumed he would have a large laboratory and research group. However, his laboratory was relatively small. Furthermore, he only had three postdoctral fellows (Anthony DeFranco, John Kung, and Maureen Howard) at my first visit. Nevertheless, he was regarded as a giant in the Immunology field. Indeed, he was a real giant, and also an outstanding mentor. Bill was a person who was glad to advise or supervise researchers when asked. Thus, young scientists with big dreams wanted to work with him. Weekly morning group meetings and one-to-one meetings with Bill were always exciting and helpful, and I learned a lot from him. Everybody respected him. He was a very kind and gentle boss. He was also an extremely intelligent man with striking creativity. But, perhaps most, I liked his shyness, because I am Japanese. I studied the functional roles of IL-4, IL-5, and IL-2 for growth and differentiation of B cells. After publishing one paper in *J Immunol* and two papers in *J Exp Med*, I left NIH and returned to Japan. I missed Bill and my friends at LI very much.

## INTRODUCTION

In Japan as a physician-scientist, I had several opportunities to learn that sepsis remains a common life-threatening disorder. Patients with high serum endotoxin levels did not necessarily develop lethal shock. Furthermore, patients with low serum endotoxin levels sometimes died of septic shock. Thus, we simultaneously measured the serum levels of endotoxin and interleukin (IL)-6, because

lipopolysaccharide (LPS) induces IL-6 production *in vivo*. We found that there were at least two groups: an endotoxin shock susceptible group, characterized by high serum IL-6 level with low serum LPS level, and an endotoxin-resistant group, characrized by low serum IL-6 level with high serum LPS level. These findings indicated the presence of certain limiting factors that determined the sensitivity of patients to endotoxin shock. I learned that priming with heat-killed *Propionibacterium acnes* (*P. acnes*), a Gram-positive bacterium, or BCG increased the sensitivity of animals to the lethal effect of LPS. Thus, with Tomohiro Yoshimoto, my long-term collaborator, I studied the mechanism for how *P. acnes* increase the responsiveness of mice to LPS. We found that *P. acnes* priming rendered mice highly susceptible to the lethal effect of LPS by enhanced production of IL-1 and/or tumor necrosis factor-α (TNF-α) as well as increased responsiveness to the stimulation with IL-1 and/or TNFα.

After publishing these results (1) in 1992, I observed the very interesting phenomenon that *P. acnes*-primed BALB/c *nu/nu* mice were resistant to LPS-induced lethal shock, and instead most of them died of fulminant hepatitis through apoptosis-mediated hepatocytotoxicity. My colleagues, Haruki Okamura and Hiroko Tsutsui, demonstrated this severe liver injury was prevented by administration of a neutralizing anti-IL-18 antibody (2). These experiments were my first exposure to the unique action of IL-18, which forms the long-term target of my investigations and the main theme of this manuscript. In this review, I will initially describe animal models of LPS-induced diseases, and then describe the actions of IL-18 on T cells and other immune cells, as the major topic of the manuscript. Finally, I will compare the actions of IL-18 and IL-33 in various aspects. Pathological roles of IL-18 in various diseases, including hepatic, metabolic, inflammatory, allergic, and autoimmune diseases, are also documented in previous (3, 4) and recent (5, 6) reviews.

## ANIMAL MODELS OF LPS-INDUCED DISEASES

### Susceptibility to LPS-Induced Endotoxin Shock

Mice primed with *P. acnes* markedly increased production of IL-1 and TNFα in response to LPS. Furthermore, these mice were highly susceptible to the lethal shock-inducing effect of IL-1 and/or TNFα (1). We tried to identify the limiting cells for LPS sensitivity. As *P. acnes*-primed BALB/c *nu/nu* mice were resistant to LPS-induced lethal shock, we examined the LPS susceptibility of these mice after reconstitution with splenic T cells from wild-type mice (7). We found that BALB/c *nu/nu* mice reconstituted with T cells became highly susceptible to LPS shock after *P. acnes* treatment and systemic administration of *P. acnes* induced development of Th1 cells in wild-type mice as well as in BALB/c *nu/nu* mice reconstituted with splenic T cells (7). Furthermore, IL-12p40-deficient mice or interferon (IFN)-γ-deficient mice were highly resistant to sequential treatment with *P. acnes* and LPS (7). Thus, IFN-γ-producing Th1 cells play an important role in determining host sensitivity to LPS shock (7).

### Susceptibility to LPS-Induced Liver Injury

The liver has a potent immune system (3). It contains residential immunocompetent cells with self-renewing ability, such as liver NK cells, extrathymically developed T cells, thymically developed $CD4^+$NKT cells, expressing CD4 and NK cell markers, and a limited T-cell antigen receptor repertoire, and Kupffer cells, tissue macrophages. With my long-term colleague Kiyoshi Matsui, I demonstrated that hepatic $CD4^+$NKT cells in non-treated wild-type mice promptly produced large amounts of IL-4 and IFN-γ upon stimulation with immobilized anti-CD3 *in vitro* (8). However, administration of heat-killed *P. acnes* induced hepatic $CD4^+$NKT cells to increase IFN-γ production, but decrease IL-4 production upon anti-CD3 stimulation *in vitro* (8). These effects were attributable to the action of IL-12 from *P. acnes*-elicited Kupffer cells, suggesting a role for Kupffer cells in regulation of immune responses in the liver (3, 8). As noted above, most mice sequentially treated with *P. acnes* and LPS developed lethal shock, while the surviving mice suffered from liver injury. Meanwhile, BALB/c *nu/nu* mice sequentially treated with *P. acnes* and LPS developed severe liver injury. However, this severe liver injury was prevented by administration of a neutralizing anti-IL-18 antibody (2). Furthermore, *P. acnes*-primed IL-18-deficient mice did not develop liver injury upon LPS challenge (9, 10). However, we found that administration of IL-18-induced liver injury in *P. acnes*-primed IL-18-deficient mice by inducing Fas ligand expression and TNFα production in hepatic NK cells (3, 11). Based on these findings, we concluded that the development of thymic T cells into Th1 cells and hepatic $CD4^+$NKT cells into predominant IFN-γ-producing cells was important for induction of LPS-driven endotoxin shock and LPS-induced liver injury in *P. acnes*-primed mice, respectively (7, 8).

## OVERVIEW OF THE IL-18/INTERLEUKIN 18 RECEPTOR (IL-18R) SYSTEM

Interleukin-18 was originally designated IFN-γ-inducing factor (IGIF), because it was first identified through its capacity to induce IFN-γ production by anti-CD3-stimulated Th1 cells (2, 12).Okamura and colleagues discovered this activity in sera or liver extracts from mice sequentially treated with *P. acnes* and LPS (2, 4). Based on the homology of its amino acid sequence to that of IL-1β, and its shared β-pleated sheet structure with IL-1β (2), IL-18 was classified into the IL-1 family of cytokines (13, 14). IL-18 is produced as a biologically inactive precursor, pro-IL-18, that is localized in the cytoplasm and requires proteolytic processing for secretion as active IL-18 (2–4). In collaboration with K. Kuida (Vertex, USA), S. Taniguchi (Shinsyu University, Japan), and J. Tschopp (University of Lausanne, Switzerland), we demonstrated that cleavage of pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, respectively, depended on the action of intracellular cysteine protease caspase-1, produced in the NLRP3 inflammasome consisting of pattern recognition receptor NLRP3 (NACHT-LRR and pyrin domain-containing protein 3), adaptor molecule ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and pro-caspase-1 (15–18). However, we also found that Fas ligand treatment stimulated

Fas-expressing Kupffer cells or macrophages to produce active IL-18 in a caspase-1-independent manner, indicating the presence of some other caspase-mediated pathways for IL-18 secretion (11). A recent study revealed that Fas mediated noncanonical IL-1β and IL-18 maturation *via* caspase-8 (19). In addition, IL-18 can be activated in an inflammasome-independent manner by proteases, such as proteinase 3 (20), chymase (21), and granzyme B (22).

Interleukin 18 receptor is composed of the inducible IL-18Rα chain (IL-1R-related protein or IL-1R5) and constitutively expressed IL-18Rβ chain (IL-1R-associated protein-like or IL-R7) (4, 6). The IL-18Rα and IL-18Rβ chains are members of the IL-1R family, and their cytoplasmic domains contain a TLR/IL-1R (TIR) domain, a common domain shared by toll-like receptors (4–6, 13, 14). IL-18Rα is an IL-18-binding receptor that, upon stimulation with IL-18, forms an IL-18 high-affinity binding heterodimer with IL-18Rβ that mediates intracellular signal transduction (23, 24). The cytoplasmic TIR domains of the IL-18R complex interact with myeloid differentiation factor 88 (MyD88), a signal adaptor containing a TIR domain, *via* a TIR–TIR interaction (4–6, 13, 14, 25). In collaboration with S. Akira (Osaka University), we revealed that the major biological activities of IL-18 were completely abrogated in MyD88-deficient mice (25). In turn, MyD88-induced events resulted in successive activation of nuclear factor-κB and mitogen-activated protein kinase by association with the signal adaptors IL-1R-associated kinase (IRAK) 1, IRAK4, and TNF receptor-activated factor 6, respectively (25), eventually leading to expression of appropriate genes, such as *Il4*, *Il13*, *Ifnγ*, *Tnf*, and *Fasl*, involved in cell differentiation, growth, survival, and apoptosis (2–6, 8–14, 23–26).

Interleukin-18-dependent cell activation can be inhibited at least by two distinct molecules. One is the naturally occurring IL-18-binding protein (IL-18BP) (27). Because IL-18BP binds to IL-18 with high affinity (400 pM), it can downregulate IL-18-induced cell responses, such as IL-18-induced Th1 cell IFN-γ production. Another inhibitor is the anti-inflammatory cytokine IL-37, a member of the IL-1 family of cytokines (28). Although IL-37 binds to IL-18Rα with low affinity, the resulting complex inhibits recruitment of IL-18Rβ, thereby abolishing signal transduction *via* IL-18R. Furthermore, this complex induces recruitment of IL-1R8, an orphan receptor of the IL-1 family formerly known as SIGIRR, to form a tripartite complex (IL-37/IL-18Rα/IL-1R8), which does not bind MyD88, but instead induces anti-inflammatory signal into the cell. Thus, IL-18 activity is inhibited by these two distinct inhibitors (6).

## MECHANISM FOR LPS-INDUCED LIVER INJURY IN *P. acnes*-PRIMED MICE

Consistent with a previous report (29), wild-type mice primed with *P. acnes* developed dense granulomas in the liver. These mice also developed acute liver injury and elevated serum IL-18 level after challenge with a sublethal dose of LPS (2–5). Although *P. acnes*-primed IL-18-deficient mice exhibited dense granulomas, similar to the liver of *P. acnes*-primed wild-type mice, they did not develop liver injury after LPS treatment (10, 11). In contrast, MyD88-deficient mice primed with *P. acnes* showed very poor hepatic granuloma formation and produced an undetectable level of IL-18 upon LPS challenge (17). This failure to produce IL-18 in response to LPS was not caused by a loss of potential of MyD88-deficient Kupffer cells to produce IL-18, because MyD88-deficient Kupffer cells were able to secrete IL-18 in response to LPS *in vitro* (30). Thus, *P. acnes* treatment induced hepatic granuloma formation in a MyD88-dependent manner and LPS stimulated Kupffer cells to produce IL-18 in a MyD88-independent manner (**Figure 1**). Next, we examined the contribution of TRIF (TIR domain-containing adapter inducing IFN-β) to *P. acnes*-induced hepatic granuloma formation and LPS-induced IL-18 secretion. In contrast to MyD88-deficient mice, *P. acnes*-primed TRIF-deficient mice showed normal development of hepatic dense granuloma, but did not release IL-18 and, therefore, did not develop liver injury (17). Thus, we concluded that *P. acnes* treatment induced hepatic granuloma formation in a MyD88-dependent manner and that subsequent LPS challenge induced caspase-1 activation in a TRIF-dependent manner in the NLRP3 inflammasome and induced IL-18 release, eventually leading to liver injury (17) (**Figure 1**).

## SEVERAL TOPICS FOR THE UNIQUE FUNCTIONS OF IL-18

### IFN-γ Production

Consistent with its original discovery as an IFN-γ-inducing factor, IL-18 can induce IFN-γ production by natural killer (NK) cells and Th1 cells that express IL-18R (2, 4) (**Figure 2**). However,

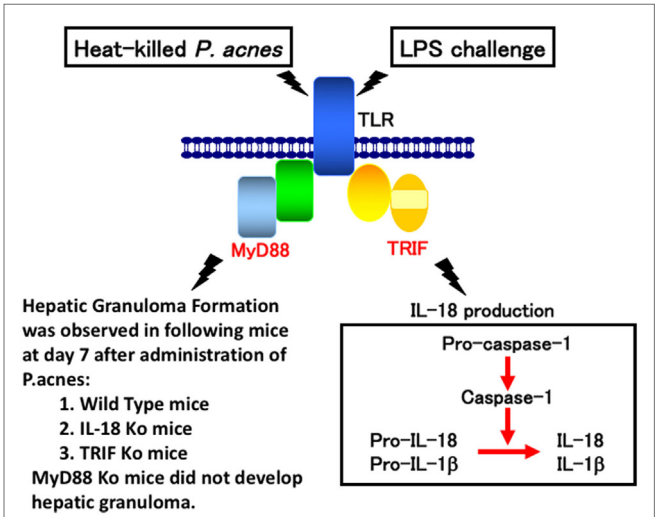


**FIGURE 1** | A proposal model for lipopolysaccharide (LPS)-induced liver injury in *Propionibacterium acnes* (*P. acnes*)-primed and LPS-challenged mice. Wild-type mice or mice deficient for interleukin (IL)-18, MyD88, or TRIF were administered with heat-killed *P. acnes* and examined for their hepatic granuloma formation at day 7 after this treatment. Only *P. acnes*-primed MyD88 did not develop hepatic granuloma at day 7 after treatment, suggesting that *P. acnes* treatment induces hepatic granuloma in a MyD88-dependent manner, but TRIF-independent manner. Although TRIF-deficient mice normally developed hepatic granulomas after *P. acnes* treatment, they could not release IL-18 or develop liver injury, suggesting that LPS TRIF-dependently activated caspase-1 *via* NLRP3 inflammasome. And, resultant IL-18 induces liver injury by induction of interferon-γ, FasL, and tumor necrosis factor-α.

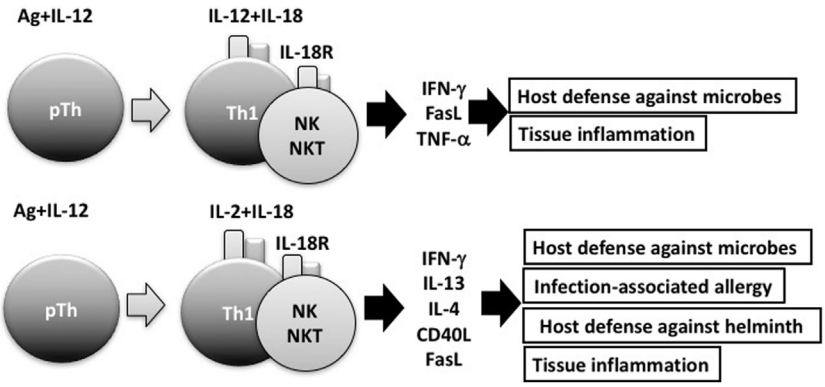


**FIGURE 2** | Interleukin (IL)-18 facilitates both Th1 response and Th2 response. In combination with IL-12, IL-18 stimulates Th1 cells, natural killer (NK) cells, and NKT cells to produce robust interferon (IFN)-γ, resulting in the clearance of intracellular microbes. However, without IL-12, but with IL-2, IL-18 induces Th1 cells, NK cells, and NKT cells to produce IL-13 and IFN-γ both of which are involved in host defense response or in infection-associated allergy. NKT cells stimulated with IL-2 and IL-18 express CD40L and produce IL-4, thereby inducing B cells to produce IgE.

IL-18 also synergizes with IL-12 to induce marked IFN-γ production by various cell types, including nonpolarized T cells, NKT cells, dendritic cells, macrophages, and B cells, through reciprocal induction of expression of their corresponding receptors (4). It is well known that B cells produce IgG1 and IgE when stimulated with anti-CD40 and IL-4. To our surprise, a combination of IL-12 and IL-18 inhibited IL-4-dependent IgG1 and IgE production, but enhanced IgG2a production by inducing IFN-γ production in B cells stimulated with IL-12 and IL-18 (31). Indeed, IL-12-stimulated B cells expressed IL-18R and strongly produced IFN-γ in response to IL-18, particularly in association with IL-12 (23). We also found that naïve Th cells stimulated with antigen (Ag) and IL-12 or IL-4 developed into IL-18R-expressing Th1 or ST2-expressing Th2 cells, respectively (23, 24, 32). Thus, expression of IL-18R and ST2 can be a convenient cell marker for Th1 and Th2 cells, respectively.

## Th2 Cytokine Production by Mast Cells and Basophils Stimulated With IL-18

In 1989, Marshall Plaut and Bill Paul reported in *Nature* that, upon cross-linkage of FcεR1 with Ag/IgE complex, mast cells, and basophils produce Th2 cytokines, including IL-4 and IL-13 (33). Thus, I was interested to know whether mast cells and basophils also had the potential to produce IFN-γ after stimulation with IL-12 and IL-18. I discussed this matter with Bill, and he said "I am very interested in what will happen." Thus, Tomohiro and I started collaboration with Bill. We found that basophils and mast cells derived by culture of bone marrow cells with IL-3 for 10 days expressed the IL-18Rα chain and produced large amounts of IL-4 and IL-13 in response to stimulation with IL-3 and IL-18 (34). These were unexpected results, but turned out to be very important findings. To our disappointment, however, mast cells and basophils never produced IFN-γ in response to various combinations of IL-3, IL-18, and IL-12 (34). As the combination of IL-18 and IL-3 stimulated basophils and mast cells to produce histamine and Th2 cytokines, we speculated that IL-18 could induce allergic inflammation without assistance from the Ag/IgE complex. Thus, we reported a new aspect of IL-18 as an inducer of Th2 cytokine production from basophils and mast cells in 1999 (34) (**Figure 3**). Later, I became interested in the capacity of basophils to produce IL-4 upon cross-linkage of FcεR1 with Ag/IgE complex. Surprisingly, we detected expression of MHC class II molecules on basophils (35). Thus, we examined the capacity of basophils pulsed with Ag/IgE complex to induce development of naïve Th cells into Th2 cells. We found that basophils had the capacity to induce development of Th2 cells (35). Although we were still unable to determine the physiological role of basophils as APCs, we believe that further studies will demonstrate such an activity in basophils.

## Innate-Type Allergic Inflammation

After publication of the paper on Th2 cytokine production by basophils and mast cells stimulated with IL-3 and IL-18, I speculated that IL-18 may have the potential to induce IL-4 production by $CD4^+$ T cells and/or $CD4^+$NKT cells. I found that injection of a mixture of IL-12 and IL-18 increased serum IgE levels in helminth-infected IFN-γ-deficient mice. Most surprisingly, daily administration of IL-18 in particular with IL-2 induced a marked increase in serum IgE levels in a $CD4^+$ T cell- and IL-4/IL-4R/STAT6-dependent manner (36). Furthermore, $CD4^+$NKT cells stimulated with IL-2 and IL-18 increased their CD40 ligand expression and IL-4 production. In addition, these activated $CD4^+$NKT cells induced development of B cells into IgG1- and IgE-producing cells. Consistent with these findings, transgenic mice overexpressing human caspase-1 in keratinocytes, established by Hitoshi Mizutani (Mie University), produced IL-18 and IgE in their sera, and also spontaneously developed atopic dermatitis (AD)-like skin lesions (37). Disruption of STAT6, required for IL-4 signal transduction, abolished IgE production without affecting the skin manifestations. In contrast, disruption of IL-18 in caspase-1 transgenic mice diminished their chronic dermatitis almost completely, although they still produced significant amounts of IgE. Thus, overproduction of IL-18 by keratinocytes induced AD-like skin lesions even in the absence of IgE and IgG1 (37). Based on these results, we designated this IL-18-induced allergic inflammation an innate-type allergic inflammation.

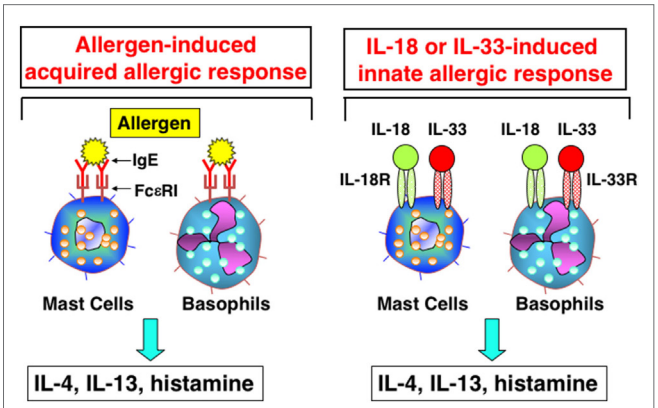


**FIGURE 3** | Interleukin (IL)-18 and IL-33 induce innate allergic response by stimulation of mast cells and basophils. Like mast cells or basophils stimulated with Ag/IgE complex, mast cells and basophils, expressing IL-18R and IL-33R, produce IL-4, IL-13 and chemical mediators, after being stimulated with IL-18 and IL-33, respectively.

In the presence of IL-2, but absence of IL-12, IL-18 stimulated NK cells, $CD4^+$ NKT cells, and splenic $CD4^+$ T cells to produce IL-3, IL-9, and IL-13 (26, 36) (**Figure 2**). Because IL-3 and IL-9 induce mucosal mastocytosis, we examined whether the animals developed mucosal mastocytosis after treatment with IL-2 and IL-18. We found that C57BL/6 mice pretreated with IL-18 and IL-2 developed mucosal mastocytosis with high levels of serum mMCP1, an activation marker of MMC, and became able to promptly expel the intestinal nematode *Strongyloides venezuelensis*. Thus, IL-18 is important for expulsion of intestinal nematodes by induction of mucosal mastocytosis, and we published these results in *J Exp Med* (38).

## Th1 CELLS PRODUCE IFN-γ AND IL-13 IN RESPONSE TO Ag AND IL-18

It is well established that IL-18 increases IFN-γ production by anti-CD3-stimulated Th1 cells, particularly in association with IL-12 (2, 4). Furthermore, endogenous IL-18 is required for host defense against intracellular microbes, such as *Listeria monocytogenes*, *Cryptococcus neoformans*, and *Leishmania major*, because IL-18-induced IFNγ activated the infected macrophages sufficiently to kill these pathogens (4, 39, 40). However, we had not examined the possibility that IL-18-stimulated Th1 cells can produce Th2 cytokines. Thus, we stimulated established ovalbumin (OVA)-specific Th1 cells with OVA and/or IL-18 and found that OVA plus IL-18-stimulated Th1 cells produce both a Th1 (IFN-γ) and Th2 cytokines (IL-9, IL-13) (41) and additional IL-2 stimulation enhanced production of Th2 cytokines (**Figure 2**).

Next, we examined whether IL-18 acts on memory Th1 cells to induce airway inflammation and airway hyperresponsiveness (AHR) in naïve host mice. In 2002, Nobuki Hayashi and Bill Paul developed a method to establish both resting Th1 and Th2 memory cells (42). Nobuki performed a wonderful study after coming back to my laboratory from the LI. To avoid a background response of host-derived T cells, he administered newly polarized OVA-specific Th1 or Th2 cells into naïve mice and allowed them to adopt a resting memory phenotypy *in vivo*. Intranasal administration of OVA induced airway inflammation and AHR only in mice that received Th2 cells (41). However, mice that received Th1 cells developed airway inflammation and AHR after intranasal administration of both OVA and IL-18 (41). Th1 cells stimulated with OVA and IL-18 became harmful cells, which we designated "super Th1 cells," that produced IFN-γ and IL-13, the combination of which induced difficult bronchial asthma (41). Nobuki further demonstrated that naïve mice having resting Th1 memory cells developed severe bronchial asthma in response to nasal administration of OVA plus LPS instead of IL-18. He also revealed that endogenous IL-18 from LPS-stimulated bronchial epithelial cells was responsible for inducing severe bronchial asthma. He published these results in 2007 (43). This prominent feature of IL-18 can explain the mechanism for infection-associated allergic diseases (44) (**Figure 2**).

Intriguingly, after several rounds of stimulation with Ag, IL-2 plus IL-18, Ag-specific Th1 cells were found to differentiate from cells producing both IL-13 and IFN-γ into cells producing IL-13, but little IFNγ. My colleague Masakiyo Nakahira verified that GATA3 was essential for induction of IL-13 in Th1 cells after stimulation of these cells with Ag, IL-2, and IL-18 (45). Thus, IL-18 has the potential to induce plasticity of established Th1 cells (41, 43–45) (**Figure 2**).

## SIMILARITIES AND DIFFERENCES BETWEEN IL-18 AND IL-33

Interleukin-33, a member of the IL-1 cytokine family, is a ligand of ST2. IL-33 is synthesized as a full-length active form, stored in the nucleus, and released from cells when they receive mechanical damage or become necrotic (46–48). IL-18 is an immunoregulatory cytokine (4) that acts with IL-12 to stimulate Ag-stimulated Th1 cells to produce IFN-γ (2, 4, 12), but acts with IL-2 to stimulate the same cells to produce both a Th1 (IFN-γ) and a Th2 cytokine (IL-13) (41, 43–45) (**Figure 2**). In contrast, IL-33 has the capacity to induce Ag-stimulated Th2 cells to increase production of Th2 cytokines (IL-4, IL-5, and IL-13) (46–48), suggesting that IL-33 plays an important role in induction of allergic responses.

We found that mast cells and basophils express both IL-18R and IL-33R and produce IL-4 and IL-13, when stimulated with IL-3 plus IL-18 or with IL-33, respectively (34, 49) (**Figure 3**). Therefore, IL-18 and IL-33 have very similar effects on mast cells and basophils. Moreover, IL-18 and IL-33 show similar pathological effects on the lungs. Nasal administration of IL-2 and IL-18 induced AHR, pulmonary eosinophilia, and goblet cell hyperplasia in wild-type mice, but not in Rag2-deficient mice (50) (**Figure 4**). However, nasal administration of IL-33 induced the same changes in both wild-type mice and Rag2-deficient mice (49) (**Figure 4**). Thus, IL-2 plus IL-18 induced these pulmonary changes in a NKT cell-dependent manner, while IL-33 treatment induced the same changes in a NKT cell-independent and innate cell-dependent manner (**Figure 4**). Moro et al. (51) and Neill et al. (52) showed that natural helper cells (NH cells) or nuocytes, currently designated group 2 innate cells (ILC2s), express IL-33R, and produce IL-5 and IL-13 in response to IL-33.

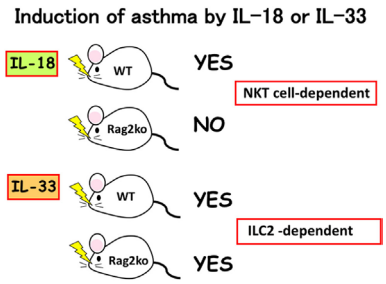


**FIGURE 4** | Induction of bronchial asthma by intranasal administration of interleukin (IL)-18 or IL-33. As natural killer (NK)T cells constitutively express IL-18R, intranasal administration of IL-18 into wild-type mice, but not into Rag2Ko mice induced bronchial asthma by induction of IL-4 and IL-13 from NKT cells. In contrast, intranasal administration of IL-33 into wild-type mice and Rag2Ko mice equally induce bronchial asthma, because Rag2Ko mice are equipped with ILC2 which express IL-33R and produce IL-13 in response to IL-33.

My long-term colleague Koubun Yasuda revealed the mechanism for how IL-33 induced the above pulmonary changes in the absence of acquired immunity. He showed that IL-33 treatment increased the number of ILC2s and that the IL-33-activated-ILC2s induced pulmonary eosinophilia and goblet cell hyperplasia by producing IL-5 and IL-13 in a T-cell-independent manner (53). Thus, IL-33 plays an important role in the induction of ILC2-dependent allergic diseases. Furthermore, he found that infection with the intestinal nematode *S. venezuelensis*, which transiently migrates into the lungs, increased the number of IL-33-producing alveolar epithelial type II cells in the lungs of wild-type mice and Rag2-deficient mice (53). Thus, both types of mice infected with *S. venezuelensis* developed eosinophilic inflammation and goblet cell hyperplasia in their lungs (Loeffler syndrome) (53). Therefore, IL-33 production and release in the lungs is very important for induction of pulmonary eosinophilic inflammation during nematode infection (53–55).

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# Distinct Immunologic Properties of Soluble Versus Particulate Antigens

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Antigens in particulate form have distinct immunologic properties relative to soluble antigens. An understanding of the mechanisms and functional consequences of the distinct immunologic pathways engaged by these different forms of antigen is particularly relevant to the design of vaccines. It is also relevant regarding the use of therapeutic human proteins in clinical medicine that have been shown to aggregate, and perhaps as a result, elicit autoantibodies.

**Keywords: bacteria, antibody, particle, vaccine, polysaccharide, autoantibody, marginal zone, antigen presentation**

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## DIFFERENCES BETWEEN SOLUBLE AND PARTICULATE ANTIGENS RELEVANT TO THE IMMUNE RESPONSE

Relative to soluble antigens, antigens in particulate form are selectively internalized through antigen-presenting cell (APC) phagocytosis, with greater efficiency (1, 2) but with longer processing time (3), exhibit quantitative and qualitative differences in the antigenic epitopes generated (4), concentrate for extended periods within the marginal zone of the spleen (5), and are presented poorly, if at all, by splenic B cells (4), although efficiently internalized by peritoneal B1b cells (6). Signaling responses of APC can differ markedly in response to microbe-associated molecular patterns that are expressed in particulate versus soluble form (7). Various particulates, including intact bacteria activate the inflammasome resulting in production of IL-1β (8, 9), a cytokine that can augment T cell-dependent antibody responses (10). Phagocytosis of particulate antigens by APC also augment the calcineurin/NFAT signaling pathway resulting in a higher level of immune stimulation (7).

## ANTIGEN PARTICULATION IMPROVES VACCINE IMMUNOGENICITY

The aggregation or particulation of an antigen could increase its overall immunogenicity by enhancing B cell receptor cross-linking, leading to higher levels of B cell activation and targeting of internalized antigen to lysosomes with subsequent enhanced antigen presentation to T cells. Thus, DNA vaccination with plasmids encoding for weakly immunogenic GFP protein fused to either a long polyQ domain that triggers aggregation or a short polyQ domain that does not resulted in a significantly higher anti-GFP antibody response to the GFP aggregate, relative to its non-aggregated form, as well as to enhanced CTL activity (11). The attachment of several vaccines to bacterium-like particles derived from the Gram-positive bacterium *Lactococcus lactis* that was treated to become a predominantly peptidoglycan shell (1–2 μm in diameter), has demonstrated significant enhancement in $CD4^+$ T cell responses, and promotion of antigen cross-presentation for $CD8^+$ T cell activation (12). Nanoparticles can also be used as a platform for synchronizing delivery of antigens and adjuvants that can be targeted to specific cell types (13). The particle size to which antigen is associated may play a critical factor in the subsequent immune response. Thus, intradermal immunization of mice with ovalbumin (OVA) covalently attached to a range of

carboxylated polystyrene microspheres (0.02–2 μm in diameter) in the absence of adjuvant demonstrated the highest OVA-specific T cell and antibody responses when using 0.04 μm, but not larger, beads (14). The immune response using 0.04-μm beads was also higher relative to OVA immunizations using a number of different adjuvants. A subsequent study from this group further demonstrated a greater uptake by lung APC, and higher immune mediator release, following intratracheal instillation in mice of 0.05-μm, relative to 0.5-μm, diameter polystyrene nanoparticles (15). In this regard vaccines, in which recombinant proteins are displayed as virus-like particles, such as hepatitis B and human papilloma virus have proven clinically safe and highly effective in preventing the corresponding viral infections (16, 17). Many additional vaccination approaches using antigen particulation as a platform are currently under investigation (18–21), to mention only a few.

One underlying mechanism involved in the adjuvant effect of particulation is the targeted delivery of antigens to APCs in a concentrated form. We demonstrated that dendritic cells (DCs) were >5,000 times more efficient in the uptake and presentation of a bacterial protein to antigen-specific T-cells when delivered on the bacterial surface than when in soluble form, as a polysaccharide (PS)–protein conjugate (1). This likely reflected the fact that ingestion of a single bacterial particle by an APC effected the uptake of multiple copies of the associated protein. In contrast, the amount of soluble protein internalized *via* pinocytosis depended more heavily on the local concentration of antigen. Thus, the same total amount of soluble PS–protein conjugate delivered at a higher concentration was internalized 10–50 times more efficiently by the DC (1). PS–protein conjugates include several licensed clinical vaccines, such as that for *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, that are highly protective against infections with these PS-encapsulated extracellular bacteria (22).

## ANTIGEN PARTICULATION CAN BREAK IMMUNE TOLERANCE

The immune system typically develops tolerance to self-proteins, yet autologous proteins used for therapeutic purposes often elicit antibody responses (23–25). Unwanted consequences of the latter include a reduction in drug efficacy (25) or development of significant pathologies (24, 26). Although the mechanism that underlies this break in immune tolerance is uncertain, possible contributors include degradation, modification, or aggregation of the protein (27, 28), or its contamination with Toll-like receptor (TLR) ligands (29, 30). In particular, aggregation has been implicated in immune responses to intravenous immunoglobulin, human growth hormone, and interferon α2 formulations (27, 28, 31, 32). Therapeutic proteins can aggregate in response to various stressors, such as agitation, freezing, and exposure to the air–liquid interface, during their manufacture, storage, and/or delivery to patients (33). Such aggregates may contain different secondary and tertiary structures that expose different epitopes, as well as create a repeating antigenic array for higher avidity B cell receptor binding and cross-linking. Indeed, immunization of rabbits or mice with virus-like particles to which arrays of self-antigens were conjugated induced strong antibody responses to those self-antigens (34, 35).

In light of the above, we directly tested the hypothesis that particulation of a soluble self-protein, i.e., mouse serum albumin (MSA), may lead to the breaking of self-tolerance in non-autoimmune mice, manifested by induction of $CD4^+$ T cell-dependent antigen-specific antibody responses. This question was directly relevant to the fact that human serum albumin has a wide variety of clinical applications including intravascular volume expansion (36) and stabilization of protein therapeutics and vaccines (37). Certain properties of albumin would suggest a low likelihood of its eliciting autoantibodies. Thus, it exhibits limited polymorphism, including no known phenotypic variation in inbred mouse strains (38). In humans, although the gene for albumin is highly polymorphic, variations in the encoded protein sequences are rare (39). Moreover, during its synthesis, albumin is non-glycosylated, reducing its potential variability, although 6–15% may undergo nonenzymatic glycation in the blood (40, 41). Other properties of albumin, however, might suggest its potential for acting as an autoantigen. Thus, glycation alters the conformation and function of albumin (42). Albumin also binds various serum ligands (43) and interacts with a variety of host cells (44) and some bacterial pathogens (45–47). Bacteria can also bind albumin indirectly such as specific binding to heme that contains bound albumin (48).

In light of the potential for therapeutic proteins to aggregate as well as the observation that albumin can bind to intact bacterial surfaces, we wished to determine whether MSA covalently attached to bacteria-sized (1 μm) latex beads could induce an autoimmune response in non-autoimmune BALB/c mice. We observed that bead-associated, but not soluble MSA was indeed able to induce a $CD4^+$ T cell-dependent MSA-specific IgG response (49). When MSA and PS (a T cell-independent antigen), were both covalently attached to the same latex beads, but not to each other we observed a $CD4^+$ T cell-dependent augmented primary, and boosted secondary IgM and IgG anti-PS response. No such effects were observed for beads linked to PS alone or with MSA beads mixed with soluble PS. These responses were enhanced by, but did not require TLR stimulation. These results provided a potential mechanism, i.e., protein aggregation/particulation for the induction of responses to self-proteins normally unable to induce specific T cell or antibody responses. Thus, measures to minimize aggregation of proteins used for therapeutic purposes may lead to a reduction in elicitation of neutralizing or pathogenic antibodies. These data further confirmed our earlier demonstration using 1-μm beads with associated PS and a *foreign* protein (50) that *non-covalent* association of protein and PS was sufficient to elicit T cell-dependent anti-PS responses. The simple association of PS and a foreign protein to a biocompatible particulate substrate might serve as a more cost-effective alternative to the use of PS–protein conjugate vaccines in which the antigens require covalent linkage, especially in developing countries where financial cost may be a limiting factor for widespread usage (51).

## PS EXPRESSED AS A SOLUBLE PS–PROTEIN CONJUGATE VERSUS THE SAME PS EXPRESSED BY AN INTACT BACTERIUM ELICITS PS-SPECIFIC ANTIBODY RESPONSES FROM DISTINCT B CELL SUBSETS AND WITH DISTINCT IDIOTYPES

Parenteral injection of particulate, in contrast to soluble, antigens results in their initial and prolonged concentration within the splenic marginal zone where they come into extended contact with marginal zone B (MZB) cells (52, 53). MZB cells, along with B-1 B cells play a major role in eliciting anti-PS responses (54). Thus, we wished to determine whether MZB cells mediated anti-PS responses to PS-expressing intact bacteria and whether or not this was also true for soluble PS–protein conjugates [the IgG anti-PS responses in both cases were shown to be $CD4^+$ T cell-dependent (55, 56)]. For this purpose we utilized $Lsc^{-/-}$ mice. The function of the Lsc protein is to attenuate Gα12/13-mediated G protein-coupled receptor signaling with subsequent activation of RhoA signaling (57). Mice genetically deficient in Lsc ($Lsc^{-/-}$) exhibit a marked defect in MZB migration from the marginal zone following immunization, precluding MZB interaction with $CD4^+$ T cells (58). Lsc acts selectively on MZB cells (58, 59).

$Lsc^{-/-}$ mice were immunized and boosted i.p. with intact, inactivated *S. pneumoniae* expressing the type 14 capsular PS or with a soluble conjugate of type 14 PS and the *S. pneumoniae*-derived cell wall protein, pneumococcal surface protein A. $Lsc^{-/-}$ mice exhibited a nearly complete abrogation in the primary and secondary IgG anti-PS responses to intact *S. pneumoniae*, whereas no effects were observed on the same IgG anti-PS response to the soluble PS–protein conjugate (1, 60). In contrast, neither the T cell-independent IgM anti-PS responses to *S. pneumoniae* nor the T cell-dependent IgG anti-protein responses to *S. pneumoniae* or soluble PS–protein conjugate were affected in $Lsc^{-/-}$ relative to control mice. Thus, these data strongly suggested that particulation of associated PS and protein selectively recruited MZB cells to induce a T cell-dependent IgG anti-PS response. This was further supported by our observation that the IgG anti-PS response to a soluble PS–protein conjugate became completely dependent on MZB cells when the conjugate was adsorbed to the surface of an intact *S. pneumoniae* that lacked *natural* expression of both the relevant PS and protein (1).

The selective utilization of MZB cells for the IgG anti-type 14 PS response to intact *S. pneumoniae* was reflected in the observation that the majority of the elicited PS-specific IgG expressed a dominant idiotype, designated 44.1-Id that was not observed when using a soluble conjugate of type 14 PS and protein (61). The idiotype of an antibody is defined as the epitope(s) within the variable region that uniquely defines the specificity of the antibody for its cognate antigen. Of note, attachment of the soluble conjugate to 1-μm latex beads or to the surface of an intact *S. pneumoniae* lacking the relevant PS and protein referred to earlier, resulted in a switch to significant 44.1-Id expression in the elicited IgG anti-PS response. Usage of the 44.1-Id was linked to the $Igh^a$, but not $Igh^b$, allotype. These results indicated that different antigenic forms of the same capsular PS can recruit distinct B cell clones expressing characteristic idiotypes under genetic control, and strongly suggested that the 44.1-Id is derived from MZB cells.

## ANTIBODY RESPONSES TO SOLUBLE ANTIGENS INVOLVE DISTINCT APCs RELATIVE TO ANTIGENS EXPRESSED BY INTACT BACTERIA

Little is known regarding the specific APCs that initiate T cell activation during T cell-dependent (TD) antibody responses to soluble antigens versus complex particulate antigens, such as inactivated, intact extracellular bacteria. Of note, aluminum salts ("alum") are often used as adjuvants when immunizing with soluble antigens in various experimental systems, and are themselves particulate. However, antigen adsorbed to alum does not behave as a particulate antigen (1). Thus, DC exposed to alum-adsorbed antigen exhibited facilitated antigen uptake, but did not internalize the alum particles themselves (62).

Dendritic cells, monocytes (and monocyte-derived cells), and macrophages, all of which can serve as APCs, are members of the mononuclear phagocyte system that can be distinguished phenotypically (63, 64). Collectively, they play dominant roles as APCs for $CD4^+$ T cells (63). Mouse DC within the spleen are further divided into conventional (classical) (c)DC [either $CD8\alpha^+$ or $CD11b^+$] and plasmacytoid DC (63, 65–67). Although DC are efficient in uptake of soluble antigens, they also exhibit phagocytic activity. Mouse monocytes are classified as $Ly6C^{hi}$ ("classical monocytes") and $Ly6C^{lo}$ ("non-classical monocytes") (63, 68). $Ly6C^{hi}$ monocytes, in particular can internalize and transport antigen to secondary lymphoid organs such as the spleen, where they mature into APCs capable of activating naïve T cells. They are then referred to, generally as monocyte-derived cells (63, 69, 70). Monocyte-derived cells appear to be especially efficient in capturing intact bacteria (71). Macrophages are highly efficient at phagocytosis and play a major role in clearing senescent and apoptotic cells, cellular debris, and pathogens, but are also capable of acting as APC to activate T cells (72). In mouse spleen, macrophages are further divided into red pulp macrophages (73), marginal zone macrophages, and marginal metallophilic macrophages, the latter two located within the MZ (52).

In light of the observation that uptake of intact bacteria and soluble antigens by APCs are skewed toward phagocytosis versus endocytosis or pinocytosis, respectively, we predicted that injection into mice of clodronate-containing liposomes (CL) (74, 75), which are internalized and toxic to highly phagocytic cells, would inhibit $CD4^+$ T cell-dependent IgG responses to antigens expressed by intact bacteria but not isolated soluble antigens. Splenic macrophages and monocytes (and monocyte-derived cells), but not conventional DCs or neutrophils, were depleted by i.v. injection of CL (76). Surprisingly, injection of CL markedly inhibited protein-specific IgG responses to a soluble PS–OVA conjugate or OVA alone, as well as to intact, inactivated

*S. pneumoniae*. In both instances, CL-mediated inhibition of protein-specific IgG responses was associated with a significant reduction in the formation of germinal centers and the differentiation of $CD4^+$ T cells into germinal center T follicular helper cells. However, CL injection which largely abrogated the proliferative response of adoptively transferred OVA peptide-specific transgenic $CD4^+$ T cells in response to immunization with *S. pneumoniae* expressing OVA peptide, did not inhibit T cell proliferation in response to soluble PS–OVA or OVA alone. In this regard, monocyte-derived cells depleted by CL, internalized *S. pneumoniae in vivo*, whereas in contrast $CD11c^{low}$ DCs, unaffected by CL injection, internalized soluble OVA. *Ex vivo* isolation and coculture of these respective APCs from *S. pneumoniae*- or OVA-immunized mice with OVA-specific T cells, in the absence of exogenous antigen, demonstrated their selective ability to induce T cell activation. These data provided strong support to the notion that distinct APCs initiate $CD4^+$ T cell activation in response to antigen expressed by intact bacteria versus antigen in soluble form. However, CL-sensitive cells appear necessary for the subsequent IgG responses to both forms of antigen (76).

These studies using CL are consistent with earlier studies demonstrating a significant CL-mediated reduction in TNP-specific IgG antibody-forming cells following i.v. immunization with other micron-sized, particulate antigens including TNP-sheep red blood cells (77) or TNP-*Lactobacillus acidophilus* (78), or reduction in serum titers of IgG anti-human serum albumin in response to liposome-associated human serum albumin (79). However, these studies provided no mechanistic basis for these observations. Similarly, i.p. injection of CL resulted in a marked inhibition in priming of $CD4^+$ T cells, including IFN-$\gamma^+$ T cells, following i.p. infection with live *Salmonella typhimurium* that was associated with a reduced accumulation of monocyte-derived cells in the spleen (80). However, in contrast to our findings, CL had no effect on the *S. typhimurium*-induced IgG2a plasma blast response and both monocyte-derived cells and conventional DC from *S. typhimurium*-infected mice could activate *S. typhimurium*-specific $CD4^+$ T cells *ex vivo*, in the absence of exogenous antigen (80). The use of a live Gram-negative bacterium in this former study, as opposed to a Gram-positive, heat-killed bacterium used in this study, may potentially underlie the observed differences. Of note, i.v. injection of CL failed to inhibit humoral immune responses to smaller, nanometer-sized particles (i.e., inactivated rabies virus or immune-stimulating complexes containing rabies virus antigens) immunized *via* the i.v. route (81). Collectively, these data add further support to the notion that antigens in particulate form have distinct immunologic properties relative to soluble antigens.

## CONCLUSION AND FUTURE DIRECTIONS

Antigens expressed in particulate/aggregated form exhibit distinct immunologic properties relative to corresponding antigens in soluble form. Cells with high phagocytic activity selectively internalize particulate antigens and do so with relatively high efficiency. Antigen within the particle is displayed in multiple copies facilitating high avidity multivalent B cell cross-linking resulting in higher and sustained levels of B cell activation and antigen internalization for presentation to $CD4^+$ T cells. This promotes higher antibody responses to foreign proteins but also a higher likelihood of generating autoantibody-secreting cells. Antigen particulation also allows for coexpression of adjuvant and cell targeting moieties for more efficient and/or targeted immune responses. Particulation itself may further activate the inflammasome and provide intrinsic adjuvant activity. Finally, particles may localize to the splenic marginal zone that may facilitate engagement of MZB that express specialized functional properties. An understanding of the unique immunologic properties of antigens in particulate form should guide future design of vaccines and protein therapeutics.

The following unanswered questions merit further study: (1) how do conventional B cells extract and present antigens from intact bacteria or protozoans in light of their inability to phagocytose particles of $\geq$1 μm size, (2) what is the significance of the differential usage of select APCs in response to soluble versus particulate antigens on the subsequent nature of the immune response, (3) what is the mechanism by which particulate or aggregated antigens break immunologic tolerance, (4) what are the precise features (e.g., size, composition, organization) of particulate antigens that lead to optimal immune responses, and (5) can directing antigen to MZB cells through particulation be exploited clinically to alter the quantity or quality of the immune response.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# Foxp3$^+$ T Regulatory Cells: Still Many Unanswered Questions—A Perspective After 20 Years of Study

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T regulatory (Treg) cells were discovered more than 20 years ago and have remained a topic of intense investigation by immunologists. The initial doubts about their existence were dissipated by the discovery in 2003 of the lineage specific transcription factor Foxp3. In this article, I will discuss some of the questions that I believe still need to be answered before we will be able to fully apply Treg therapy to the clinic. The major issue that remains to be resolved is how they mediate their suppressive functions. In order to correct defective suppression in autoimmune disease (assuming it is a causative factor) or to augment suppression in graft versus host disease or during organ transplantation, we still need to fully understand the biochemical nature of suppressor mechanisms. Similarly, in cancer, it is now widely accepted that reversal of Treg suppression would be highly desirable, yet which of the many purported pathways of suppression are operative in different tumors in different anatomic sites. Many of the concepts we have developed are based on *in vitro* studies, and it remains unclear if these concepts can readily be applied to Treg function *in vivo*. Our lack of a specific cell surface marker that readily allows us to identify and target Treg *in vivo*, particularly in man, remains a major stumbling block. Finally, I will review in some detail controversies regarding the origin of Treg, thymus versus periphery, and attempts to reverse Treg suppression by targeting antigens on their cell surface, particularly members of the TNF receptor superfamily. Hopefully, these areas of controversy will be resolved by in depth studies over the next few years and manipulation of Treg function will be placed on a more solid experimental footing.

**Keywords: T regulatory cells, tolerance mechanisms, Foxp3, suppression mechanisms, autoimmune diseases**

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## WILLIAM E. PAUL: IN MEMORIAM

I first met Bill Paul in 1971 at an extremely low point in my career. I was looking for a new supervisor for my postdoctoral training as I had just spent about 18 months working in a lab where I had accomplished absolutely nothing. Bill had just been appointed Chief of the Laboratory of Immunology, was quite understanding of my situation, and advised me to speak with Ira Green about potential opportunities in his lab. Ira took me on as postdoc and pointed me in the right direction. Bill also assumed a co-supervisory role particularly on projects that he and Ira had studied together for many years dealing with the function of immune response genes. I thrived in this environment and after only two full years as a postdoc was offered a tenured position in the Laboratory of Immunology where I have remained for the past 45 years. My lab and Bill's lab were immediately adjacent to each other on the 11th floor of the Clinical Center and we had numerous interactions on a daily basis. For

over 20 years we had joint data and journal clubs for our groups every Wednesday and Friday morning. One fringe benefit of these discussions was that my postdoctoral fellows benefited from Bill's wisdom and criticism. His comments were always delivered in a gentle fashion often pointing out major areas of deficiency or steps in the wrong direction. The fellows always accepted them and never felt threatened as they were always perceived as constructive. I am not certain my comments on his fellow's presentations were always similarly perceived! When we began our studies on T regulatory cells even I was somewhat leery as to how Bill would react to our attempts to redefine T suppression after its death in the 1980s. Bill was actually quite receptive of our approach and continued to encourage me to continue even after I received a negative review from our advisory committee. He was particularly proud to announce to the committee that I received the William Coley Award in 2004 for our studies on regulatory T cells in spite of their negative comments.

## INTRODUCTION

In 2002, I wrote a review entitled "$CD4^+CD25^+$ Suppressor T Cells: More Questions Than Answers (1)." Foxp3 had yet to be discovered as the marker for this lineage and the term "Regulatory" rather than "Suppressor," had not yet become the convention. Over the past 15 years, this field has seen tremendous growth and the therapeutic manipulation of T regulatory (Treg) function has reached the clinic. Certain aspects of the field that have received great attention and many of the questions I posed in 2002 have been answered. However, some questions remain unanswered and our lack of knowledge of these aspects of the field in my view has clearly hindered progress in the clinical application of Treg either to boost their function in autoimmunity or disable their function in malignancy. In this review, I will focus on several questions that I believe remain unanswered.

## ASSAYS OF Treg FUNCTION *IN VITRO*

My group (2) and the Sakaguchi group (3) described the first assays for the measurement of the suppressor function of $CD4^+CD25^+$ T cells *in vitro*. Although this type of assay was rapidly adopted by almost all investigators in the field, a number of issues have emerged that render interpretation of the results of these experiments problematic. In general, these assays involve the measurement of the proliferation of mouse non-Treg cells (either $CD4^+$ or $CD8^+$) triggered by TCR signaling in the presence of a titration of highly purified Treg cells. In the original studies, soluble anti-CD3 stimulation was used to trigger the TCR and the assay was always performed in the presence of accessory cells (T-depleted spleen cells, or more recently dendritic cells) that were needed to cross-link the anti-CD3 antibody and provide co-stimulatory signals. The addition of anti-CD28 was not recommended, as it was more difficult to achieve significant suppression with greater levels of TCR stimulation. The basis for this recommendation was the observation that Treg primarily inhibited proliferation by blocking IL-2 production by the responder population and anti-CD28 enhances IL-2 production by prolonging IL-2 mRNA half-life. The initial studies attempting to adapt this assay for use with human Treg frequently incorporated anti-CD28 co-stimulation to achieve significant levels of stimulation. While suppression was observed under these culture conditions, higher numbers of Tregs were required to achieve significant suppression and ratios of 1:1 (Treg:responder) were frequently employed. However, assay conditions very similar to those used in the mouse can be used with human cells (4). Significant levels of stimulation in the absence of anti-CD28 with the most commonly used anti-CD3 antibodies (OKT3 and UCHT1) can readily be achieved when a population of $HLA\text{-}DR^+$ non-T cells are used as an accessory cell population.

A number of investigators questioned the use of the soluble anti-CD3 and accessory cell approach and claimed that the use of a defined number of anti-CD3 coated or anti-CD3 and anti-CD28 coated beads was a much more precise method for stimulating T cell activation. Although Tregs are capable of inhibiting responses induced by this activation protocol, suppression again almost always required 1:1 or at best 1:2 ratios of Treg to responder cells and no suppression was frequently seen at lower ratios of Treg to responder cells. A number of questions can be raised about the use of antibody bound to beads or anti-CD3 coated plates. T cell stimulation by antibody coupled to solid surfaces may result in a qualitatively distinct signal from stimulation induced by antigen presented on professional APC or even soluble anti-CD3 stimulation in the presence of APC. In our initial studies in the mouse on Treg suppression *in vitro* (2), we found that it was exceedingly difficult to suppress T cell stimulation induced by plate bound anti-CD3. Furthermore, this resistance to suppression was not overcome by using lower concentrations of anti-CD3 to coat the plate. Our interpretation of this result was that fewer T cells were triggered to proliferate at lower concentration of plate bound antibody, but that every T cell that bound to the solid phase stimulus still received a potent signal which was resistant to Treg-mediated suppression. This question has yet to be resolved and the use of a two cell assays versus a three cell assay remains controversial.

The second issue raised by these experiments is the cellular target of Treg-mediated suppression. One of the simplest explanations for our failure to achieve significant suppression with solid phase coupled stimuli is that the target of Treg-mediated suppression *in vitro* is not the responder T cell but the APC. A wide variety of cell types have been described as direct targets of Treg-mediated suppression (**Table 1**), yet after 20 years of study,

**TABLE 1** | Cellular targets for $Foxp3^+$ T regulatory-mediated suppression.

| |
|---|
| $CD4^+$, $CD8^+$ T cells |
| Dendritic cells |
| B cells |
| Macrophages |
| Osteoblasts |
| Mast cells |
| NK cells |
| NK T cells |
| Adipocytes |
| Endothelial cells |
| Fibroblasts |
| Muscle |
| Hair follicle stem cells |

it remains unclear whether the APC or the responder T cell or both are targeted by Tregs in the widely used *in vitro* suppression assay. While multiple mechanisms of Treg-mediated suppression have been proposed (see below), suppression of APC function or delivery of APC-derived co-stimulatory signals have achieved the greatest attention. If the APC is the primary target for Treg suppression *in vivo*, it would be ideal to employ an *in vitro* assay that would mimic the *in vivo* action of Treg.

## Treg DEFECTS IN AUTOIMMUNE DISEASE

Why is it important to have a reliable *in vitro* assay for Treg suppressor function? It has been proposed and in fact widely accepted that defects in Treg function play an important role in the pathogenesis of autoimmune disease in man (5). While some early studies claimed that patients with certain autoimmune diseases had a decreased percentage or even absolute number of Treg in their peripheral blood, the overwhelming consensus today is that patients with autoimmune diseases have normal numbers of Treg at least in their circulation. A defect in numbers in target organs remains possible, but difficult to assess in man. It therefore follows that Tregs from patients with autoimmune diseases must be functionally abnormal. The number of autoimmune diseases with purported defects in Treg function as detected *in vitro* has recently been summarized by Grant et al. (6). Defects in virtually all the common autoimmune diseases including SLE, MS, T1D, RA, autoimmune thyroid disease, psoriasis, IBD, primary biliary sclerosis, autoimmune hepatitis, and primary sclerosing cholangitis have been described. Indeed, it would be difficult to publish a paper claiming normal Treg function in any of these diseases. There are a number of reasons for defective Treg suppression *in vitro* in autoimmune disease:

1. Environmental—the production of pro-inflammatory cytokines by APC such as IL-6 (7) which can provide a potent co-stimulatory signal for T effector cell expansion and render the responder T cells resistance to suppression. IL-6 could also act on Treg cells and reverse their suppressive function or result in their conversion to Th17 cells.
2. T effector cell intrinsic resistance to suppression.
3. Treg intrinsic defects including defective generation, survival, stability, or altered TCR repertoire. Finally, specific defects in one of the proposed mechanisms of Treg-mediated suppression.

While dissection of which of these factors are operative in a given autoimmune disease is clearly doable in a well-characterized animal model, in human disease in the presence of normal numbers or percentages of Treg cells, one must rely on *in vitro* assays of suppressor function. The question to be addressed is whether *in vitro* suppression assays are capable of detecting major or even minor alterations in Treg function that mimic their defective function *in vivo*. The approach I have used to begin to address this question is to ask whether defects in Treg suppression *in vitro* can be detected with Treg cells derived from mice who develop autoimmune disease secondary to a deletion or mutation of a given gene specifically in Treg cells [Traf3 (8), CD28 (9), id2/id3 (10), ubc13 (11), Itch (12), NF-κB p65 (13), Helios (14), ThPoK/LRF (15), A384Tmutant of Foxp3 (16), and EZH2 (17)]. The thymic development of Treg is normal in all these strains and all have normal numbers of Treg cells; while all have moderate to severe autoimmune disease, but all have normal Treg suppressor function *in vitro*. Notably, when tested, Treg from many of these strains exhibit abnormal function *in vivo* in their capacity to suppress the adoptive transfer of IBD in immunodeficient mice following the transfer of naïve T cells. In a number of other studies of mouse strains with selective deletion of genes in Treg cells and resultant manifestation of severe autoimmunity [Bach2 (18), satb1 (19), IRF-4 and Blimp1 (20), and LKB1 (21)], the investigators have not even bothered to test Treg suppressor function *in vitro*.

What factors could account for the failure of *in vitro* suppression assays to detect defects in Treg suppressor function? The Foxp3$^+$ Treg population is heterogeneous and can be broadly subdivided into a naïve/quiescent/resting cell subpopulation and into a memory/effector/activated subpopulation. These two populations in the mouse can be distinguished by the differential expression of CD44 (22) or Ly-6C (23). The memory/effector subpopulation (CD44$^{hi}$, Ly-6C$^-$) appears to undergo increased TCR signals *in vivo* based on increased levels of CD5 expression and CD3ζ phosphorylation (23). Most importantly, the memory population contains a high percentage of cycling cells (~10%/day) based on Ki-67 staining (**Figure 1**). By contrast, when analyzed *in vitro*, Treg are characterized as anergic or non-responsive and fail to proliferate when stimulated with anti-CD3 alone, when stimulated with combinations or anti-CD3 and anti-CD28, or with high concentrations of IL-2 (2). The memory phenotype subpopulation manifests much higher suppressive activity *in vivo* (23). Furthermore, deletion of TCR expression from Treg results in a selective loss of the cycling MP Treg combined with a loss of Treg-mediated suppressor function *in vivo* (24). Thus, one major distinction between Treg function *in vitro* versus *in vivo* is the failure to see proliferating Treg under any culture conditions

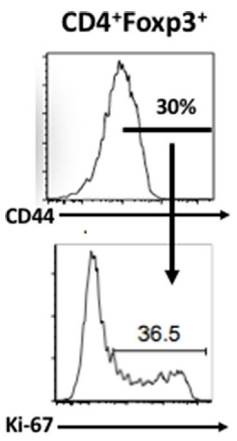


**FIGURE 1** | T regulatory Treg cells represent one of the most active cycling lymphocyte populations *in vivo*. After gating on Foxp3$^+$ T cells, we then gated on the activated/effector/memory subset as define by high levels of CD44 expression. The CD44$^{hi}$ population was then stained for Ki-67 expression. Ki-67 positivity reflects cell division over the previous 48-h period.

*in vitro*. It is quite possible that the activated/memory/effector Treg do not survive *in vitro* and their function is, therefore, never actually measured in standard *in vitro* assays. As the proliferating memory phenotype Treg are the major suppressive population *in vivo*, the relationship of what we observe in suppression assays *in vitro* to their physiologic suppressive function vivo remains unclear. While this conclusion is primarily based on studies with mouse Treg cells and human Treg cells may manifest different properties, I remain skeptical that we can use *in vitro* assays to define a defect in Treg suppressor function in autoimmune disease in man.

## MECHANISMS OF Treg-MEDIATED SUPPRESSION

One of the fundamental questions that one can raise regarding defects in Treg function is which mechanism of Treg-mediated suppression is actually defective? I have summarized (**Figure 2**) many of the proposed pathways by which Treg may manifest their suppressor effector function including release of soluble suppressor factors, cytolysis, disruption of metabolic pathways, and pathways used to selectively target DCs. The prevailing view in the field is that there is not one universal pathway by which Treg mediate suppression and that Treg have the luxury of picking from this large list of mechanisms to find one (or more) suitable for a particular situation or inflammatory niche. In fact, there are very few *in vivo* studies clearly supporting this hypothesis. One common mistake is that neutralization of a given pathway, for example, blocking the action of IL-10 (25) or TGF-beta with resultant loss of suppression, indicates that Treg are using only that pathway to mediate suppression. The alternative explanation is that the contribution of these suppressor cytokines is necessary, but not sufficient, for Treg-mediated suppression. Thus, in the xeno-graft versus host disease (GVHD) model (26) production of TGF-beta by Treg is required for prevention of disease, but Treg could also using other pathways at the same time. Indeed, Treg production of TGF-beta may only be required under "super-inflammatory" conditions such as xeno-GVHD, as mice with a selective deletion of TGF-beta in Treg do not exhibit an autoimmune phenotype (27). A similar scenario can be proposed for the requirement of IL-10 production for Treg-mediated protection from IBD, but not for the much less inflammatory autoimmune gastritis where IL-10-deficient Treg are fully protective (28).

The leading candidate for the most predominant suppressor mechanisms utilized by Treg is the downregulation of the expression of CD80/CD86 expression on DCs which is mediated

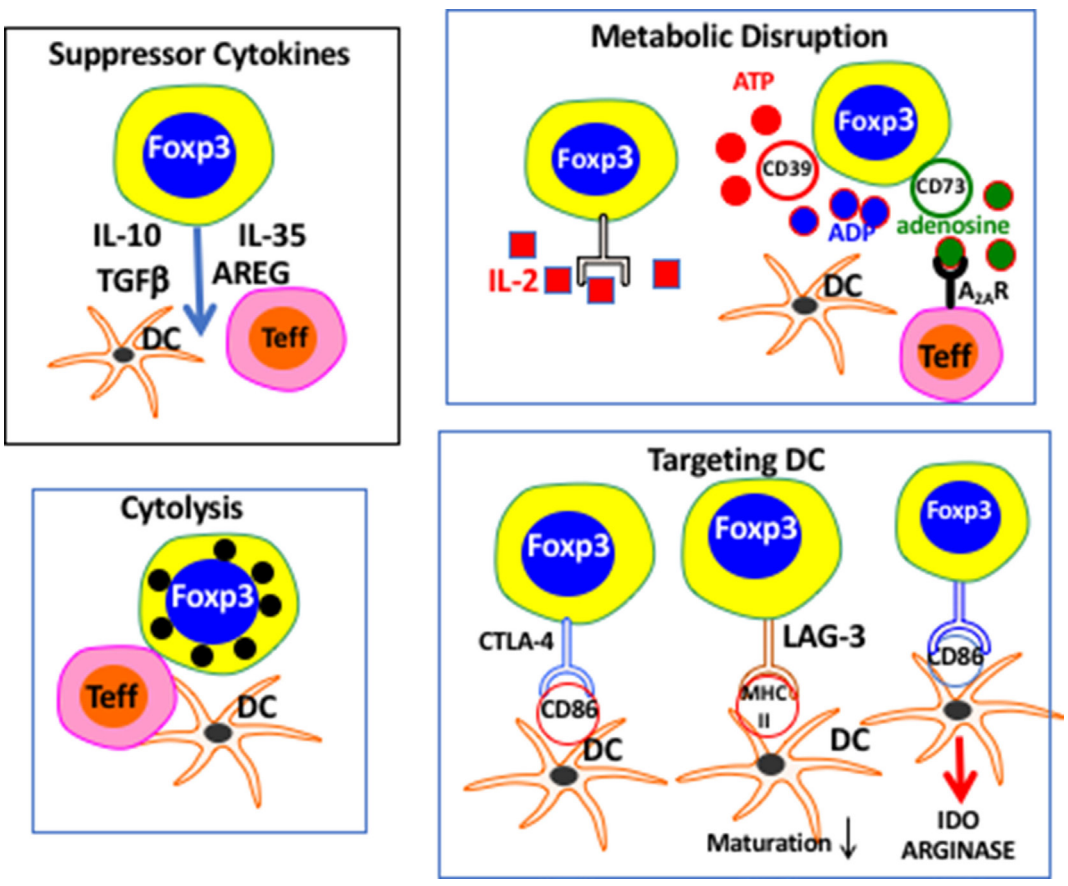


**FIGURE 2** | Proposed pathways of T regulatory (Treg)-mediated suppression. The pathways are roughly divided into different mechanistic categories. It remains unclear which or how many mechanisms are used by Treg under physiologic conditions *in vivo*.

by CTLA-4 expressed on Treg cells. It was first noted that Treg were the only lymphocyte population that expressed CTLA-4 constitutively and several early studies demonstrated that Treg suppression could be reversed *in vitro* (29) and *in vivo* (30) by anti-CTLA-4. This model received strong support for the studies of Wing et al. (31) which demonstrated that selective deletion of CTLA-4 expression from Treg resulted in the rapid development of autoimmune disease. Furthermore, Qureshi et al. (32) demonstrated that CTLA-4 was capable of selectively removing CD80/CD86 from the cell surface of DCs by a process of transendocytosis ultimately resulting in the degradation of CD80/CD86 within the Treg. Taken together these studies appear to offer a solid experimental foundation that this pathway is the major one utilized by Treg. However, several more recent studies suggest that the function of CTLA-4 in Treg is considerably more complex. First, it should be pointed out that in the studies of Qureshi et al. (32), CTLA-4 on activated conventional T cells could also mediate the transendocytosis of CD80/CD86. Thus, this pathway is not specific for Treg. Second, the recent studies of Paterson et al. (33) which demonstrated that specific deletion of CTLA-4 from the adult mouse Treg resulted in enhanced Treg proliferation *in vivo* and was accompanied by increased Treg suppressor function *in vivo*. Similarly, we have observed (34) that the homeostatic proliferation of Treg *in vivo* can be markedly enhanced by treatment of mice with anti-CTLA-4. The enhanced proliferation of Treg in this model was accompanied by enhanced proliferation of memory phenotype $CD4^+$ and $CD8^+$ T cells consistent with a loss of Treg suppressor function. Thus, after almost 20 years of intensive study, the role of CTLA-4 in Treg function remains unclear.

The second pathway of Treg-mediated suppression that deserves further discussion is whether consumption of IL-2 by Treg plays any role in Treg-mediated suppression. When we first presented the results of our Treg suppression assays in one of our joint lab meetings some 20 years ago, Bill's first reaction was that they must be inhibiting by functioning as "IL-2 sinks" a concept originally proposed in the early 1980s (35). We always took Bill's advice seriously and were then obligated to rule out this mechanism. We demonstrated that Treg inhibited proliferation by blocking the induction of IL-2 mRNA production in the responder T cell (2) and this observation was confirmed by many groups (36). The one exception being the studies of Pandiyan et al. (37) who claimed that Treg consume IL-2 and inhibit the proliferation of $Foxp3^-$ T cells leading to Bim-mediated apoptosis. A number of observations have biased me against the concept of the "IL-2 sink" as an important pathway of Treg-mediated suppression: (A) It is widely assumed that because Treg express high levels of CD25 that they have high number of high affinity IL-2 receptors. In fact, no one has determined the number of high-affinity IL-2 receptors on Treg and it is likely that while they probably express in the range of 50,000 CD25 molecules that they express at least a log lower CD122 and CD132 molecules resulting in a level of expression of the high affinity IL-2R (the tri-molecular complex) similar to that seen on activated $Foxp3^-$ $CD4^+$ T cells. (B) The addition of exogenous IL-2 has no effect on Treg-mediated suppression of IL-2 production by $CD4^+$ $Foxp3^-$ T cells at the mRNA level (38). (C) In a trans-species model where human Treg can efficiently suppress mouse responder cells, the addition of a blocking anti-human CD25 had no effect on the suppressive function of the human Treg (4). (D) While IL-2 is critical for T cell proliferation and expansion *in vitro*, the expansion of $CD4^+Foxp3^-$ T cells *in vivo* in response to antigen stimulation occurs in the absence of IL-2 signaling, as antigen-specific T cells lacking CD25 expression expand as well as wild-type T cells following antigen recognition (28).

In addition to potentially functioning as an "IL-2 sink" for the inhibition of T effector proliferation, IL-2 may also play a critical role to support the maintenance of Foxp3 expression, Treg survival, and Treg proliferation by triggering the STAT5 pathway. However, it should be noted that the Treg subpopulation that appears to be responding to IL-2 homeostatically is the resting Treg population, not the activated cycling population suppressive population. In our studies, IL-2 played no role in Treg cycling *in vivo* (22). Chinen et al. (39) have attempted to resolve some of these issues by deleting expression of CD25 from Treg in combination with the expression of a constitutively active form of STAT5. The expression of the active form of STAT5 rescued mice from the autoimmune disease present in the CD25 deficient mice. These studies revealed that expression of CD25 on Treg was not needed for suppression of $CD4^+$ responder T cells, but IL-2 consumption by CD25 expressed on Treg played a major role in suppression of $CD8^+$ T cells. One explanation for this dichotomy is that $CD8^+$ T cells are more sensitive to IL-2 signaling than $CD4^+$ T cells. While these elegant genetic studies appeared to resolve the issue of IL-2 consumption at least for suppression of $CD4^+$ T cell responses, more recent studies have shown that Treg cells expressing phospho-STAT5 localize in clusters in lymph nodes with IL-2 producing $CD4^+$ $Foxp3^-$ T cells (40). This localized response of Treg to IL-2 signaling also appeared to enhance their suppressive function. Thus, while deprivation of $CD4^+$ effector T cells of IL-2 by Treg may not be play a role in suppression, the action of IL-2 locally produced by T effectors on Treg may be critical for their optimal suppressive activity presumably mediated by pathways other than IL-2 consumption. Indeed, we demonstrated over a decade ago that the initial production of IL-2 by responder T cells was required to activate the suppressor function of Treg which in turn suppressed the subsequent production of IL-2 by responder T cells (38).

## ANTIGEN-SPECIFIC SUPPRESSION VERSUS POLYCLONAL SUPPRESSION *IN VIVO* AND *IN VITRO*

One of the major conclusions drawn from studies of Treg suppressor function *in vitro* using both polyclonal Treg cells and antigen-specific Treg cells is that following stimulation *via* their TCR, the suppressor effector function of Treg is completely antigen non-specific. Thus, once activated by their cognate antigen, Treg specific for antigen A could suppress the proliferation of T effectors specific for antigen B (41). This concept is supported by studies which demonstrated that antigen-specific Treg cells are more potent inhibitors of disease than polyclonal Treg (42). However, our understanding of the mechanisms of

Treg-mediated suppression *in vivo* is in a less advanced stage that our understanding of Treg-mediated suppression *in vitro*. A number of fundamental questions need to be addressed including: (1) the site of suppression (target organ or lymphoid tissues), (2) do Tregs inhibit homing of effector cells to the target organ, (3) can polyclonal Treg migrate to the target organ, (4) does suppression *in vivo* require the continuous presence of the Treg, (5) is suppression reversible, or (6) has a permanent state of tolerance been induced. None of these questions has definitively been answered and solutions are needed for the development of rational Treg therapies. Most importantly, we need reductionist models *in vivo* that will allow each aspect of the activation of T effector cell response to be analyzed. The field appears to be satisfied with studies demonstrating defective Treg suppressive activity in the classic cell transfer model of induction of IBD using polyclonal Treg, as originally described by Powrie and collaborators (43). However, this model is very complex as disease may be mediated by different T effector subsets (Th1 or Th17), involves both anti-self and anti-non-self responses as contribution of the intestinal microbiome is critical. Very few studies have addressed how Treg with defects in transcription factor function or signaling pathways actually fail to mediate suppression *in vivo*.

## REVERSAL OF Treg-MEDIATED SUPPRESSION

I have already discussed the significance of neutralizing Treg suppression with antibodies to suppressor cytokines. An extension of this approach to dissecting mechanisms of Treg-mediated suppression has been to reverse suppression with antibodies to cell surface antigens expressed on Treg cells that play a role in the process of suppression. We (44) and others (45) first described that polyclonal or monoclonal antibodies to a member of tumor necrosis receptor superfamily, the GITR (TNFRSF18), could reverse Treg-mediated suppression *in vitro*. However, this conclusion was rapidly drawn into question as $CD4^+$ $Foxp3^-$ T cells can also express the GITR and more importantly expression of the GITR is rapidly upregulated on $Foxp3^-$ T cells following TCR activation. Indeed, when we cultured combinations of WT and $GITR^{-/-}$ Treg and effector T cells, we only observed reversal of suppression when the GITR was expressed on the responder T effector cells (46). Thus, engagement of the GITR on T effector cells by an agonistic antibody rendered the responder T cells resistant to suppression. It is highly likely that a similar induction of resistance to suppression in T effector cells is responsible for the purported reversal of Treg suppressor function (47) by agonistic antibodies to OX40 (CD137).

The most prominent member of the TNFRSF family that has been implicated in Treg function is TNF itself. Several studies with human T cells have reported that TNF could inhibit the function of Treg and that anti-TNF treatment of patients with RA resulted in restoration of defective Treg function when measured *in vitro* (48). However, TNF has also been demonstrated to have potent co-stimulatory function on T effector cells and it is likely that the TNF may have exerted its function on the T effectors rendering them resistant to suppression in a manner similar to the studies in the mouse with anti-GITR. Recent studies have failed to reproduce the deleterious effects of TNF on Treg function and have actually demonstrated that exposure of human Treg to TNF increased their expression of CD25 and Foxp3 (49).

It remains possible that future studies may identify cell surface antigens on Treg that are involved in Treg-mediated suppression. Hopefully, such studies will result in the development of agonistic antibodies that can either selectively expand Treg, enhance or alternatively reverse their suppressive function. While the studies discussed above were based on the enhanced expression of several members of this family on Treg (GITR, OX40, and TNFRII), the effects of these reagents *in vitro* and probably *in vivo* were mediated by their action as co-stimulatory molecules for T effector cells. Although this is a valuable lesson to have learned, it also has potentially clinical applications. In animal models, antibodies to the GITR have been shown to partially deplete Treg *in vivo* in the tumor microenvironment and to simultaneously provide co-stimulatory signals to $CD4^+$ and $CD8^+$ T effector cells resulting in inhibition of tumor growth (50). The usefulness of such reagents in the clinic remains to be evaluated.

## tTreg, pTreg, iTreg, AND ex-Tregs

The concept that Treg cells could only be generated in the thymus was challenged by studies in the mid-2000s (51, 52) which demonstrated that Treg cells could be generated both *in vivo* (pTreg) and *in vitro* (iTreg) from peripheral $CD4^+$ $Foxp3^-$ T cells. TGF-beta plays a prominent role in the process, particularly *in vitro*. While there is little dispute about both of these phenomena, the significance, size, and function of the pTreg pool remains to be fully characterized. A significant impediment to progress has been a lack of a defined marker for thymus derived (tTreg). We have suggested that Helios is a useful marker of tTreg (53). Other groups have suggested that neuropilin-1 (Nrp1) is a more useful and more specific marker (54). There are important differences in the expression of these two antigens. First, Helios is a transcription factor thereby limiting its usefulness for isolation, although we now have generated a faithful Helios reporter mouse. Helios is expressed by 70–80% of Treg in peripheral lymphoid tissues and by a somewhat lower percentage (50–60%) of mucosal derived Treg. By contrast, Nrp1 is expressed by 85% of peripheral Treg. The mAb generated against mouse Helios cross-reacts with human Helios and reacts with 80% of Treg in human peripheral blood. Both Helios and Nrp1 can be expressed by conventional T cells in the mouse, although we have not been able to detect Helios expression in human non-Treg under any conditions *in vivo* or *in vitro* (55). The expression of Nrp1 by human Treg is unclear. One major deficiency of using Nrp1 as a marker of tTreg is that its expression is regulated by TGF-beta. Thus, pTreg generated in the central nervous system were shown to be suppressive, but uniformly expressed Nrp1; iTreg generated in culture in the presence of TGF-beta are uniformly $Nrp1^+$ (54). Furthermore, the percentage of $Nrp1^+$ Treg is greatly reduced in mice with a T cell-specific deletion of TGF-beta clearly demonstrating that the constitutive expression of Nrp1 is closely regulated by TGF-beta (unpublished observations).

For these reasons, we strongly favor the use of Helios as a definitive marker of tTreg. However, it is incumbent upon us to prove that this is the case. In order to study the differences between Helios$^+$ and Helios$^-$ Treg, we have generated a Helios-GFP/Foxp3-RFP double reporter mouse. The Helios$^+$ Treg population expressed a more activated phenotype and had slightly higher suppressive capability *in vitro*. Both subsets were equivalent in their ability to suppress IBD *in vivo* and both subsets expressed a highly demethylated TSDR, with slightly higher demethylation in the Helios$^+$ Treg subset. This result is consistent with the concept that pTreg generated *in vivo* are relatively stable (56). Upon transfer to normal mice, both Helios$^+$ and Helios$^-$ Treg cells maintained equal Foxp3 stability and Foxp3$^+$Helios$^+$ Treg maintained stable expression of Helios. Preliminary analysis of the TCR repertoire of both subsets by deep sequencing revealed little to no overlap of the two populations consistent with distinct origins of the subsets (unpublished observations). Taken together, our data indicate that Helios expression can differentiate two distinct populations of Treg with overlapping functions, most likely representing tTreg (Helios$^+$) and stable peripherally induced pTreg (Helios$^-$). However, considerable controversy still exists regarding the use of Helios as a marker for tTreg (57, 58) and caution should still be exerted when using this marker.

Several studies over the past 5 years have challenged the notion that Foxp3$^+$ Treg cell lineage is stable and have raised the possibility that Treg cells can lose Foxp3 expression particularly when present in an inflammatory milieu resulting in "reprogramming" of Treg to potentially pathogenic T effector cells (59). As Treg express an anti-self biased TCR repertoire, these re-programmed Treg would represent a potential potent population of T cells capable of inducing autoimmune disease. As complete deletion of Treg from adult mice results in exuberant inflammation and death in 10–15 days (60), the maintenance of Treg stability is critical to the survival of the host. For this reason, we favor the view that most tTreg are very stable and are unlikely to lose Foxp3 expression. Nevertheless, the studies of Treg instability are convincing and need to be addressed. One possibility is that the unstable population of Treg primarily develops from the pTreg population. pTregs represent logical candidates for instability even though most may have a demethylated TSDR. Alternatively, a minor population of pTreg may not be fully committed to the Treg lineage. The studies of Miyao et al. (61) clearly demonstrate the existence of a small population of Treg that can readily lose Foxp3 expression and can rapidly expand *in vivo* and thus appear to represent a large percentage of Treg in fate mapping studies. Other studies suggest that tTreg can also manifest Foxp3 instability (62). The recent demonstration (63) of a population of unstable and dysfunctional Treg in the tumor microenvironment that still maintain Foxp3 expression adds further complexity to our understanding of the role of "ex-Tregs." Studies in the future need to resolve the issue of tTreg versus pTreg and the role of Treg stability. It is unclear if the loss of Treg stability contributes to the pathogenesis of any human autoimmune diseases, but this is a difficult issue to address experimentally.

## THE FUTURE

Although many of the issues posed above have not yet been completely addressed, the use of Treg for cellular biotherapy has already reached the clinic in studies for the prevention of GVHD following stem cell transplantation (64) as well as autoimmune disease (65). The successful use of low-dose IL-2 treatment to expand Treg in two clinical trials (66, 67) has stimulated great interest. A recent perusal of ClinicalTrials.gov has revealed 181 proposed studies involving the use of Treg cells and a number of trials of low dose IL-2 treatment alone or in combination with Treg cellular therapy are planned. Of note, no studies are listed using specific pharmacologic manipulation of Treg function or using monoclonal antibodies to enhance or suppress Treg function. My own view is that the development of such reagents is required before we will have the necessary tools for the therapeutic manipulation of Treg cell function in man. As emphasized in this review, further studies of the biological properties of Treg, particularly the specific mechanisms of suppression utilized in given disease states, are needed as the foundation for the development of pharmacologic reagents.

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## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# Posttranscriptional (Re)programming of Cell Fate: Examples in Stem Cells, Progenitor, and Differentiated Cells

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How a single genome can give rise to many different transcriptomes and thus all the different cell lineages in the human body is a fundamental question in biology. While signaling pathways, transcription factors, and chromatin architecture, to name a few determinants, have been established to play critical roles, recently, there is a growing appreciation of the roles of non-coding RNAs and RNA-binding proteins in controlling cell fates posttranscriptionally. Thus, it is vital that these emerging players are also integrated into models of gene regulatory networks that underlie programs of cellular differentiation. Sometimes, we can leverage knowledge about such posttranscriptional circuits to reprogram patterns of gene expression in meaningful ways. Here, we review three examples from our work.

**Keywords: posttranscriptional regulation, RNA-binding protein, microRNA, embryonic stem cell, Th17, fetal hematopoiesis, gene regulatory network, hematopoietic stem and progenitor cells**

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## INTRODUCTION

The sequencing of the first human genome (1), in principle, provided us with a complete parts list and blueprint for building a human being. However, our work is far from done, and a quote from Richard Feynman applies here: "What I cannot create, I do not understand." This is a daunting challenge for biologists, since we know little about how all these parts fit and work together to make a functional human cell, the basic unit of life. Furthermore, it has been estimated that an average adult human being is composed of 30–37 trillion cells (2, 3). How a single-cell embryo can give rise to all these cells and ultimately a whole organism is still poorly understood.

The answer must be contained in the genome if we could fully decode it. First, the central dogma of molecular biology posits that DNA (the genome) is transcribed into RNA (the transcriptome) and then translated into protein (the proteome) (4). Thus, RNA has been considered mainly as a "messenger" to transmit information encoded in the genome to produce the proteome. However, even if we understood the function of all the proteins encoded by our DNA that would only account for ~1% of the information content of the genome (1). That leaves the bulk of the genome, presumably harboring the blueprint for life, that we are only beginning to understand. For example, a part of the blueprint that is best understood contains instructions for the transcriptional machinery to either switch genes on or off. Indeed, gene regulation at the DNA level within the cell's nucleus is an active and exciting field of research. However, it has come to light that RNA does not only serve as a template to encode protein, also known as messenger RNA (mRNA). It turns out that most of the genome (~75%) is transcribed, in other words, able to generate complementary RNA (5), but

these transcripts are not always translated giving birth to the field of "non-coding RNAs."[1] As such, the number of annotated non-coding RNAs rivals the number of protein-coding transcripts (6), and we will need to determine what functions these factors of emerging importance play. To draft an outline of a working roadmap for putting all these parts together, system biologists have begun mapping various types of networks to catalog as comprehensively as possible how diverse biomolecules interact with each other. We are doing our small part to begin integrating the roles of regulatory non-coding RNAs and associated RNA-binding proteins in this larger framework. We have noticed a recurring theme from our work (**Figure 1**).

## INDUCING PLURIPOTENCY BY POSTTRANSCRIPTIONAL REPROGRAMMING

If we knew the genetic programs underlying cell fate specification, it would be possible to instruct cells to perform desired biological functions at will. For example, Takahashi and Yamanaka employed four transcription factors to instruct mature somatic cells to de-differentiate back to an embryonic-like pluripotent stem cell state (7). Interestingly, two independent groups found that they could accomplish this feat in cellular reprogramming using a class of small (19–23 nucleotides long) non-coding RNAs called microRNAs (miRNAs) (8, 9). This represents one example of posttranscriptional reprogramming; however, the mechanisms of action are not well understood.

We have previously reported that ablation of Dicer, the RNAse III-containing enzyme required for miRNA processing impairs mouse embryonic stem cell (ESC) differentiation and self-renewal (12). Furthermore, Dicer is required for the generation of induced pluripotent stem cells (13). A reasonable candidate for mediating these activities is the miR-290 family (14), a miRNA cluster that is highly expressed in mouse ESCs and is downregulated during differentiation. Interestingly, the miR-290 locus has one of the top ranked super enhancers in ESCs (seventh out of 231), higher than the pluripotency genes encoding Oct-4 and Nanog (15). miRNAs target complementary mRNAs by base pairing, *via* their so called seed sequence, a six to eight nucleotide motif at their 5′ end (16). Members of miR-290 share the same seed sequence as the miR-302 family used in the two studies mentioned earlier and therefore are predicted to target the same mRNAs. We determined that expression of the Trithorax group protein Ash1l is posttranscriptionally repressed by these ESC-specific miRNAs (**Figure 1A**) (14). Ash1l is a methyltransferase which promotes tri-methylation of histone H3 at lysine 36 (H3K36me3), an epigenetic mark associated with ongoing gene transcription. One function of Ash1l is to antagonize Polycomb-mediated gene silencing (17). The polycomb repressive complex 2 (PRC2) catalyzes tri-methylation of H3K27, a histone mark associated with

[1]This popular term in an unfortunate misnomer because it suggests that these RNAs do not harbor genetic code. It would have been more accurate to call them untranslated RNAs rather than non-coding RNAs.

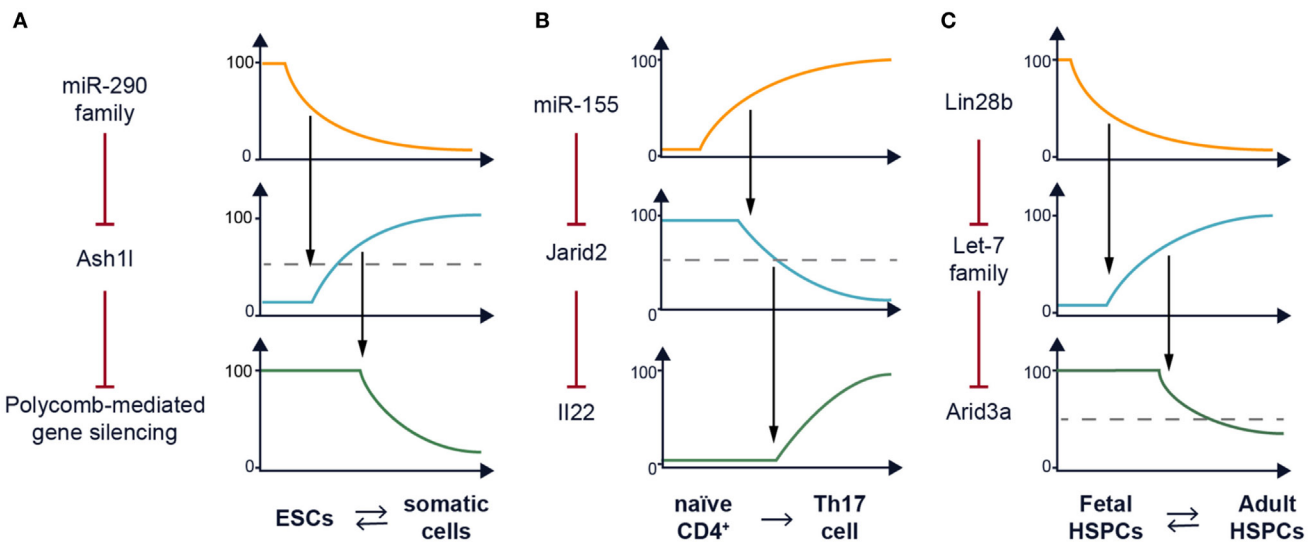


**FIGURE 1** | Recurrent network motif in posttranscriptional (re)programming. **(A)** The miR-290–Ash1l–polycomb repressive complex 2 (PRC2) axis plays a role in setting the chromatin landscape of embryonic stem cells (ESCs) to support the pluripotency gene expression program. A model of how the expression of the miR-290 family and Ash1l varies to impact activity of PRC2 is depicted along a time course as ESCs undergo differentiation. This process is reversible (7–9). **(B)** The miR-155–Jarid2 axis can also remodel the chromatin landscape by regulating PRC2 recruitment to support the Th17 gene expression program including transcription of the *Il22* cytokine gene among many others. A model of how the expression of miR-155 and Jarid2 varies to impact *Il22* transcription is depicted along a time course as naïve CD4+ T cells undergo Th17 differentiation. This process has not been shown to be reversible. **(C)** The Lin28b–let-7 axis mediates the fetal–adult hematopoietic switch. One downstream target of this pathway in B cell lineage progenitors is *Arid3a* messenger RNA which encodes a transcription factor (10). A model of how the expression of Lin28b and the let-7 family varies to impact *Arid3a* posttranscriptionally is depicted for hematopoietic stem and progenitor cells (HSPCs) during ontogeny. This process is reversible (11).

silencing. PRC2 has been shown to be essential for pluripotency maintenance and induction (18, 19). Indeed, we found that in the absence of miRNAs, the *Homeobox* (*Hox*) gene clusters, which are canonical targets of PRC2, have reduced H3K27me3 marks and PRC2 occupancy and are de-repressed. This defect in epigenetic silencing could be rescued by transfection of a single representative member of the miR-290 family (14, 20). Furthermore, this defect can also be rescued by Ash1l knockdown (14). A similar study, showing defective polycomb recruitment in the absence of miR-290, was independently performed by Graham et al. (20) further confirming the importance of this family of miRNAs in ESC pluripotency. In summary, a single miRNA family can reprogram the epigenetic landscape of a cell. By affecting the balance between H3K36me3 and H3K27me3, miR-290 can promote the pluripotent program of gene expression (**Figure 2**).

## REPROGRAMMING CHROMATIN IN Th17 CELLS

This general principle can be used in any cellular differentiation system. Indeed, we found a second similar example; although, it was not the original motivation of our work to demonstrate the generality of our idea. We screened for potentially interesting miRNAs in mouse T helper cell differentiation and found that miR-155 is highly expressed in Th17 cells compared with other subsets. Expression of miR-155 was induced upon T cell activation and was highly dependent on addition of IL-6 and IL-1β (21). Furthermore, we found that these two cytokines synergistically activated miR-155 expression in Th17 cells in a Stat3-dependent manner (21, 22), and later realized that the *Mir155* locus harbored a super enhancer (23). Our investigations further revealed that this miRNA also plays a role in programming the epigenetic landscape in Th17 cells (24). In the absence of miR-155, there is increased recruitment of PRC2 to thousands of locations in the genome, and enhanced tri-methylation of H3K27 at those sites. While Th17 cell differentiation still occurs in the absence of miR-155, we found significant defects in cytokine gene expression, a vital function of Th17 cells. In miR-155 knockout mice, we found $CD4^+ROR\gamma t^+$ Th17 cells *in vivo*, but they displayed a significant cell-intrinsic defect in IL-17 and IL-22 expression (24).

We determined that the root of the problem is de-repression of Jarid2, a target of miR-155 in Th17 cells (**Figure 1B**), and a key component of PRC2. It was recently found that Jarid2 is essential for recruitment of PRC2 to chromatin (25–29). Indeed, the defect in cytokine gene expression by Th17 cells can be rescued partially by deleting just one allele of Jarid2, thus reducing its expression by 50%. The partial rescue we observed with the compound deletion of miR-155 and Jarid2 highlights the fact that miRNAs target multiple transcripts and often it is hard to identify a single target that can restore the dysregulation of an miRNA deficiency. In that same experiment, we also observed genetic epistasis between miR-155 and Jarid2 with regards to homeostasis of $Foxp3^+$ T regulatory cells indicating that this regulatory circuit is used again in a different context. Thus, the concentration of Jarid2 can be used to modulate the global activity of polycomb-mediated gene silencing, and we have uncovered a situation in which miR-155 has co-opted this function as a rheostat.

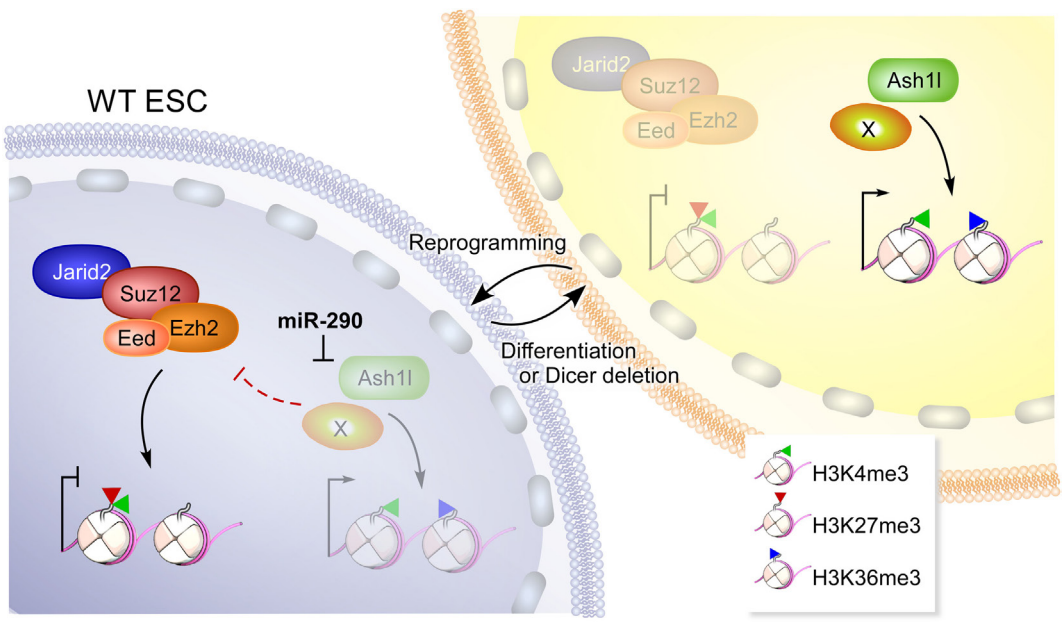


**FIGURE 2** | A model depicting how miR-290 reprograms the epigenome of embryonic stem cells (ESCs). In wild-type (WT) ESCs, high levels of miR-290 represses Ash1l and perhaps additional targets (depicted by "X") that can otherwise antagonize polycomb repressive complex 2 (PRC2) (containing Ezh2, Eed, Suz12, and Jarid2). PRC2 activity results in deposition of H3K27me3 marks on chromatin including bivalent domains that harbor H3K4me3 (20), a mark on active or poised promoters. Upon Dicer deletion or differentiation, miR-290 levels are reduced and can no longer repress Ash1l and additional targets. Unfettered, Ash1l activity results in increased H3K36me3 marks and antagonizes PRC2 (17). This figure is reproduced from Kanellopoulou et al. (14).

## Lin28b-MEDIATED REPROGRAMMING IN HEMATOPOIESIS

In a third project, we screened for miRNAs that distinguished progenitor B (pro-B) cells isolated from fetal liver versus adult bone marrow. The let-7 family of miRNAs is highly expressed in pro-B cells from adult bone marrow but not fetal liver (11). Since the different let-7 members are encoded by seven disparate genetic loci, it seems unlikely that this differential expression is regulated transcriptionally. Rather we postulated that there could be posttranscriptional regulation of the whole family. An RNA-binding protein, Lin28, had already been discovered to inhibit maturation of let-7 miRNAs (30), was a likely candidate (**Figure 1C**). In support of our hypothesis, we found that Lin28b, one of two paralogs, is highly expressed in fetal hematopoietic stem and progenitor cells (HSPCs) but not in their adult counterparts. Furthermore, enforced expression of Lin28 in adult HSPCs reprogrammed lymphocyte development to mimic fetal ontogeny. As evidence that we have uncovered a general molecular mechanism for fetal–adult hematopoietic switching, ectopic expression of LIN28B in adult erythroblasts is also sufficient to turn on fetal hemoglobin expression (31). This provides a novel avenue for the treatment of beta-thalassemia and sickle cell disease that may avoid the cytotoxic effects of hydroxyurea, currently the only clinically approved treatment for beta-globinopathies. Furthermore, we hope to inspire a new and better strategy to regenerate the hematopoietic and immune system. Specifically, Lin28b-reprogrammed HSPCs may be useful for transplantation in neonates or *in utero* if adult hematopoietic stem cells could be rejuvenated to become fetal again.

On a personal note, Bill Paul would frequently ask whether we had looked at embryonic-derived macrophages and whether their specification might also depend on Lin28b. Sadly, we failed to provide Bill with an answer before he passed away, but we are working hard to determine whether Lin28b also (re)programs myeloid lineages, in addition to lymphoid and erythroid differentiation in memory of his inquisitiveness.

## CONCLUSION

Overall, these studies support the idea that studying posttranscriptional regulatory networks will not only reveal interesting molecular mechanisms for controlling gene expression programs but can also provide novel therapeutic targets for reprogramming cell fates.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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# The Role of Molecular Flexibility in Antigen Presentation and T Cell Receptor-Mediated Signaling

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Antigen presentation is a cellular process that involves a number of steps, beginning with the production of peptides by proteolysis or aberrant synthesis and the delivery of peptides to cellular compartments where they are loaded on MHC class I (MHC-I) or MHC class II (MHC-II) molecules. The selective loading and editing of high-affinity immunodominant antigens is orchestrated by molecular chaperones: tapasin/TAP-binding protein, related for MHC-I and HLA-DM for MHC-II. Once peptide/MHC (pMHC) complexes are assembled, following various steps of quality control, they are delivered to the cell surface, where they are available for identification by αβ receptors on $CD8^+$ or $CD4^+$ T lymphocytes. In addition, recognition of cell surface peptide/MHC-I complexes by natural killer cell receptors plays a regulatory role in some aspects of the innate immune response. Many of the components of the pathways of antigen processing and presentation and of T cell receptor (TCR)-mediated signaling have been studied extensively by biochemical, genetic, immunological, and structural approaches over the past several decades. Until recently, however, dynamic aspects of the interactions of peptide with MHC, MHC with molecular chaperones, or of pMHC with TCR have been difficult to address experimentally, although computational approaches such as molecular dynamics (MD) simulations have been illuminating. Studies exploiting X-ray crystallography, cryo-electron microscopy, and multidimensional nuclear magnetic resonance (NMR) spectroscopy are beginning to reveal the importance of molecular flexibility as it pertains to peptide loading onto MHC molecules, the interactions between pMHC and TCR, and subsequent TCR-mediated signals. In addition, recent structural and dynamic insights into how molecular chaperones define peptide selection and fine-tune the MHC displayed antigen repertoire are discussed. Here, we offer a review of current knowledge that highlights experimental data obtained by X-ray crystallography and multidimensional NMR methodologies. Collectively, these findings strongly support a multifaceted role for protein plasticity and conformational dynamics throughout the antigen processing and presentation pathway in dictating antigen selection and recognition.

**Keywords: major histocompatibility complex, T cell receptor, tapasin, transporter associated with antigen presentation, TAP-binding protein, related, chaperone**

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## DEDICATION

In recognition of William E. Paul's personal encouragement to explore new approaches to address fundamental aspects of the immune response, we offer this review that reflects recent progress in studies of antigen presentation and T cell receptor-mediated signaling. Dr. Paul's commitment to rigorous analysis and quantitative experimentation continues to serve as a paradigm for our research.

## INTRODUCTION

Experimental approaches to solving fundamental problems in immunology range from the biological to the biophysical, exemplified by early observations concerning immunity to infection and chemical and biochemical studies of toxins, blood groups, haptens, and antibodies. Contemporary molecular biological and structural studies of antibodies, major histocompatibility complex (MHC) molecules, Fc receptors, and T cell receptors (TCRs), as well as many other immunologically relevant molecules, not only expand our understanding of the immune system but also have been instrumental in developing methodologies with broader application (1). Central to the immune response are the cellular pathways of antigen processing and presentation—the mechanisms by which peptides derived from foreign or self proteins are degraded into peptides of appropriate length and are then captured by MHC class I (MHC-I) or MHC class II (MHC-II) molecules which display these peptide fragments as peptide/MHC (pMHC) complexes at the surface of antigen-presenting cells (APC) (2, 3). Such pMHC complexes are then available for identification by T cells, which are subsequently activated to initiate various cellular programs. These may result in cytolysis of target cells (primarily by $CD8^+$, MHC-I-restricted T cells) or production of various cytokines (by either $CD8^+$ or by $CD4^+$, MHC-II-restricted T cells) that direct, coordinate, and induce further immunological responses such as antibody production by B cells or differentiation into memory T cells. Cell surface MHC-I molecules may also interact with various inhibitory, and in some cases activating, natural killer (NK) cell receptors, and thus contribute to a regulatory role in the NK arm of the innate immune response (4–6). Various genetic, molecular biological and structural approaches have examined peptide–protein and protein–protein interactions that are necessary to generate an immune response. Our primary goal in this review is to highlight the role of molecular flexibility in governing molecular interactions required for antigen processing, presentation, and recognition, as illustrated by the function of MHC molecules, their chaperones, and TCR in antigen presentation and recognition. Recent reviews have summarized aspects of this flexibility, largely based on computational approaches (7, 8). Our emphasis here will be on recent experimental observations based on X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (9–15).

Much of our current understanding of protein structure has been revealed by X-ray crystallography, a technique that is unrivaled in its ability to provide high resolution structural details (16–19). X-ray data often reveal regions of proteins that are found in poor electron density, or that exhibit high values of the crystallographic *B*- factor, indications of flexible or dynamic parts of the molecule (20). Computational molecular dynamics (MDs) and normal mode analysis, based on X-ray structures, provide predictive approaches to visualizing protein flexibility (7, 21, 22). However, the most informative experimental elucidation of dynamic regions of proteins comes from NMR spectroscopy. NMR analysis of proteins in solution provides information on conformational changes over time scales ranging from picoseconds to days, thus encompassing dynamics ranging from bond vibrations to side chain flips to large scale domain motions, and the residue-specific stability of H-bonds (23). NMR is also powerful because it can characterize sparsely populated (i.e., transient) conformational states that may be important for biological function (24). Contemporary protein-labeling and multidimensional NMR techniques permit examination of protein complexes as large as 1 MDa (25, 26). In addition, all atom MD simulations may complement the experimental NMR and contribute to elucidating such dynamic processes. The discussion below focuses on the dynamics of proteins involved in antigen presentation, largely based on experimental analyses.

In this review, we will explore the dynamics of pMHC with respect to three aspects of antigen presentation: (1) the formation of the tri-molecular complex consisting of peptide, and MHC [for MHC-I, peptide, MHC-I heavy chain, and the light chain, β2-microgolobulin ($\beta_2$m)] as inferred from numerous X-ray structures and recent NMR analyses; (2) the influence of the pMHC chaperones, tapasin and TAP-binding protein, related (TAPBPR) for MHC-I and HLA-DM (H2-DM in the mouse) for MHC-II; and (3) alterations of the conformational dynamics of the TCR upon pMHC interaction that reflect early steps in TCR-mediated signaling. Our focus is on MHC-I, but we will describe analogous steps in the MHC-II processing and presentation pathway as well. Our discussion of peptide, MHC-I, MHC-II, and TCR dynamics follows brief summaries of the major steps of MHC antigen processing and presentation.

## MAJOR STEPS IN MHC ANTIGEN PROCESSING AND PRESENTATION: MHC-I

The cellular and molecular bases by which peptides are generated by the proteasome in the cytoplasm, transported *via* transporter associated with antigen presentation (TAP) to the endoplasmic reticulum (ER), where they are loaded onto nascent MHC-I, have been the focus of considerable attention for several decades, and a number of reviews address this process (2, 8, 27–31). Here, we summarize the process and the critical steps, with a focus on MHC-I, as shown schematically in **Figure 1**. MHC-II follows a similar but distinct process (32, 33).

The antigenic peptides bound by MHC-I in general derive from proteins located in the cytoplasm, proteins that are degraded by the proteasome following unfolding or misfolding and ubiquitination, or proteins that result from aberrancies in translation initiation, mRNA splicing, or alternate

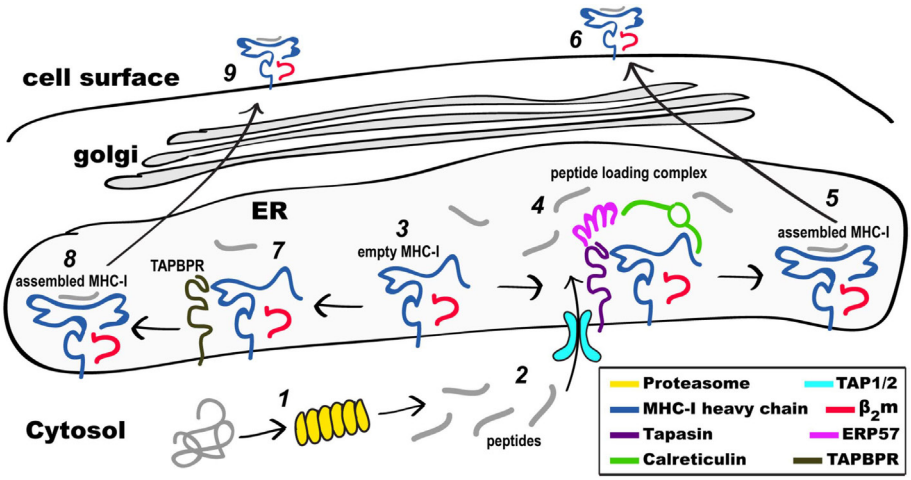


**FIGURE 1** | Schematic view of MHC class I (MHC-I) pathways of antigen processing and presentation. Proteins in the cytosol engage the proteasome (1) and the peptides generated (2) are transported through the TAP1/2 transporter to the endoplasmic reticulum (ER). Partially folded MHC-I/β2-microgolobulin ($\beta_2$m) complexes (3) are stabilized as part of the peptide loading complex (PLC) (4) where they may be retained in a peptide-receptive state. Once high affinity peptide is bound, the peptide/MHC-I (5) is released from the PLC and destined for the secretory pathway and the cell surface (6). Alternatively, partially folded MHC-I/$\beta_2$m complexes (3) are stabilized by interaction with TAP-binding protein, related (TAPBPR) (7), loaded with peptide, released from TAPBPR (8), and the assembled MHC-I proceeds to the cell surface (9). Not illustrated are the peptide trimming enzymes (ERAAP or ERAP1/2) or the quality control UGGT1 interaction as described in the text.

reading frames (34, 35). The sources of these peptides may be self-proteins, sometimes expressed at abnormal levels in cancer cells, or pathogen-derived products expressed following infection. These peptide products of the proteasome must then be transported to the site of MHC-I folding, assembly and maturation, the ER, a function provided by the heterodimeric transporter associated with antigen processing, TAP (29, 36). In the ER, peptides load onto MHC-I following motif rules during the folding process, are trimmed by aminopeptidases, and their binding is monitored by quality control mechanisms in the ER and Golgi. For MHC-I, limits on the preferred length of antigenic peptides are imposed by a binding cleft with closed ends, and peptides, usually of 8–12 amino acids in length, are generated by the progressive results of proteasome degradation, length limits for TAP transport, and amino-terminal trimming by endoplasmic reticulum aminopeptidase 1 (ERAP1).

MHC class I heavy chains, the "human leukocyte antigens," denoted HLA-A, -B, or -C in the human, H2 in the mouse, are ~40 kDa glycoproteins that exhibit the greatest genetic polymorphism known. Presently, some 13,000 HLA class I and almost 5,000 HLA class II alleles are recognized (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html). The MHC-I heavy chain, a type I membrane glycoprotein, assembles with the monomorphic light chain, $\beta_2$m, and a peptide, usually of 8–12 amino acids in length, taken from the cell's ER peptide pool (**Figure 1**). Peptides are bound *via* anchor positions that engage pockets of the MHC, designated A–F (37). Any "single" MHC molecule, purified from a cellular source, can associate with an ensemble of hundreds or thousands of self-peptides (38). The first crystal structure of an HLA-A2 molecule was based on a heterogeneous peptide/HLA-2/$\beta_2$m preparation and thus electron density corresponding to the peptide was poorly defined (39). Numerous subsequent X-ray structures have been determined based on methods for producing homogeneous complexes employing various expression and purification strategies (40).

Intracellularly, the MHC-I protein is synthesized on membrane-bound ribosomes and delivered vectorially into the lumen of the ER, where initial folding, including formation of the intrachain disulfide bond of the membrane proximal α3 domain, along with assembly to the $\beta_2$m light chain, takes place. A molecular chaperone, calnexin (41, 42), stabilizes the partially folded regions of MHC-I until the heavy chain engages the peptide loading complex (PLC), which consists of the heterodimeric TAP1/2 peptide transporter (43), tapasin (44), a chaperone that stabilizes peptide receptive (PR) MHC-I, ERp57, a tapasin-associated oxidoreductase (45), and calreticulin (46). Peptides load onto PR MHC-I in the PLC, and trimming of their amino termini is accomplished by ER-associated amino peptidase [known as ERAAP in the mouse (47) or ERAP1/2 in the human (48)]. Recently, cryo-electron microscopic images of the full PLC purified from a human lymphoblastoid cell line have been obtained (49), revealing a multimolecular complex containing the two-pseudo-symmetric editing modules, centered around the TAP transporter, consistent with previous biochemically derived structural models (27, 50). Once high affinity peptide is loaded onto MHC-I, the pMHC/$\beta_2$m complex is released and then proceeds through the Golgi where quality control based on carbohydrate composition occurs (51, 52). The acquisition of high-affinity peptide by MHC-I is assured by the coordinated functions of the proteins of the PLC, in particular tapasin. In recent years, a tapasin homolog, TAPBPR, has been recognized as a molecule with similar function to the PLC, but that accomplishes its role independent of the PLC and its associated

components (53). Assembled, stable, peptide/MHC-I (pMHC-I) complexes are then displayed at the cell surface.

## MAJOR STEPS IN MHC ANTIGEN PROCESSING AND PRESENTATION: MHC-II

The folding, assembly and peptide-loading of MHC-II molecules, though similar in some respects to that of MHC-I, occurs in distinct cellular compartments and is focused on binding peptides generated not from an "inside-out" pathway like MHC-I, but rather from those produced from proteins that derive from the extracellular environment (2, 33). Thus, proteins taken up by endocytosis or phagocytosis enter the endocytic pathway where they are proteolyzed and denatured, and where they encounter MHC-II molecules, consisting of previously assembled complexes consisting of α and β chains bound to the Ii (invariant chain). Processing of Ii, release of the CLIP peptide derived from it, and concomitant interaction with HLA-DM, a peptide-exchange catalyst/chaperone, result in peptide-loaded (PL) MHC-II that then go to the cell surface for recognition by $CD4^+$ T cells (54–56). The role of HLA-DM in optimizing the class II peptide repertoire parallels the role of the PLC or TAPBPR in the MHC-I peptide loading pathway (57).

## PEPTIDE DYNAMICS

Dynamics of peptides bound to MHC molecules have been the focus of both experimental and computational studies that have been recently reviewed (58). Characterizing peptide conformational plasticity and dynamics within the MHC groove is of considerable interest because peptides: (1) influence MHC thermal and kinetic stability as well as the structural ensembles and free energy landscape of the assembled MHCs and (2) play a key role in recognition by TCR and NK receptors (NKR). These features of molecular flexibility of peptides are important for a proper immune response and impact MHC cell surface lifetime, receptor recognition and antigen immunogenicity. Exactly how peptide dynamics regulate antigen processing and presentation is an ongoing field of study.

Association of the TCR with pMHC molecules often induces localized conformational changes in the backbone and side chain of the bound peptide (59). It is hypothesized that if the peptide is presented by the MHC with a conformation and surface chemistry that is not optimized for TCR recognition, the pMHC will exhibit slow TCR binding, relative to a peptide presented in a more restricted, pre-optimized conformation. During this antigen recognition process, peptide motions impact the formation of complementarity pMHC/TCR interaction interfaces, in terms of both shape and chemical composition. The timescale of the peptide motions contributes to the affinity of pMHC/TCR recognition by imposing energetic and kinetic barriers for complex formation, and stability of the resulting complex. Initial insights into this phenomenon were obtained from a comparison of the X-ray conformations of the HTLV-1 derived $Tax_{11-19}$ peptide bound to HLA-A2 in the presence or absence of a high affinity TCR indicated an induced fit of the peptide of the pMHC complex when bound to the TCR (58). In these structures, the conformational change in $Tax_{11-19}$ upon TCR binding is highlighted by significant rearrangements of the backbone atoms of Pro6 and Val7. NMR analyses of the $^{15}N$- and $^{13}C$-labeled $Tax_{11-19}$ peptide bound to HLA-A2 revealed multiple resonances for Val7 of the peptide reflecting a slower than millisecond timescale of interconversion between alternate peptide conformations. In this example, the crystallographic suggestion of conformational plasticity of an MHC-I-bound peptide has been reinforced by the behavior in solution as detected by NMR.

Multiple peptide conformations have also been observed in the well characterized QL9/H2-$L^d$ model system, where NMR analyses revealed two conformations of the bound 9-mer QL9 peptide as indicated by the presence of two unique chemical shifts in slow-exchange for the amide resonance of Phe7 of the peptide in the MHC-bound state (60). Intriguingly, Phe7 was reported to remain conformationally mobile even when interfacing with the CDR3β loop of the cognate 2C TCR. Matching conformational dynamics between receptor and ligand has been proposed as a mechanism to enhance the thermodynamic stability of pMHC/TCR complexes (60). This may result from reducing the entropic penalty associated with restraining otherwise flexible surfaces and reflects the enhanced stability of what might otherwise be a weak TCR/pMHC complex.

An illustration of the dynamic nature of pMHC-II molecules was seen in the pigeon cytochrome *c* (PCC) 91–104 peptide/I-$E^k$ (pMHC-II) model system in which two distinct conformations of a bound $^{19}F$-labeled peptide were observed by NMR (61). The two peptide conformations corresponded to kinetically distinct species of PCC91–104/I-$E^k$ complexes identified by their fast and slow dissociation rates (62). Careful studies of MHC-II molecules binding peptides displayed in alternate registers reveal potential complexities that may result from the peptide binding groove being open at both ends (63). Conformational isomers of the same peptide presented by the same MHC-II molecules have been identified based on distinct T cell reactivities (64). Indeed, one study employing spin-labeled peptide and NMR analysis demonstrated that an MHC-II-restricted peptide can bind in either the canonical N to C (left to right) or flipped (right to left) conformation (65).

## "EMPTY" MHC-I MOLECULES

Peptides bound in the groove of MHC-I and MHC-II molecules serve two indispensable and interrelated functions: (1) to form part of a composite pMHC ligand recognized by T and NK cell receptors and (2) to structurally stabilize MHC molecules for long-lived display at the cell surface. However, under certain physiological conditions "empty" or peptide-free conformers of MHC-I occur at the cell surface as detected by specific monoclonal antibodies or by peptide binding assays. The LA45 monoclonal antibody reacts with a $\beta_2$m-free form of human HLA molecules on phytohemagglutinin-activated human mononuclear leukocytes and on transformed cell lines (66). Similarly, in the mouse, the 64-3-7 antibody recognizes peptide free forms of H2-$L^d$ in cellular lysates and at the cell surface (67). Peptide-binding

experiments indicate the presence of empty, peptide-receptive HLA-B27 molecules on the cell surface (68), perhaps contributing to the etiology of HLA-B27-associated arthritic disease. A functional role for empty MHC-I molecules at the cell surface in modulating immune responses was inferred from early studies (69–72). A recent report identified empty HLA-B*35:01 molecules on activated T cells and showed preferential binding of such alternatively conformed structures to CD8 resulting in enhanced T cell responses (73).

MHC class I molecules devoid of, or bearing low affinity, peptides fail to reach the cell surface efficiently at physiological temperature, but can be detected if the cells are incubated at room temperature (74). The distinct conformation of such molecules may be discerned by comparing the reactivity of monoclonal antibodies that detect peptide-independent and peptide-dependent epitopes (67, 75, 76). These "empty" MHC-I molecules result from genetic lesions in the peptide-loading steps of the antigen presentation pathway, specifically in major components of the PLC including TAP and tapasin (77, 78).

Some non-classical MHC-I-like molecules, such as human HLA-F can be expressed as either peptide-free (open-conformer) or PL forms. Such molecules may differentially interact with either activating or inhibitory NKR to innate immune responses (79–81). Understanding the structural contributions of peptide to fully loaded MHC-I and MHC-II molecules provides insight into the mechanisms involved in peptide loading and exchange. However, the instability of peptide-free molecules has precluded crystallographic studies of these molecules.

Nuclear magnetic resonance methods are especially well-suited to analyzing conformational dynamics in MHC-I molecules since these proteins are routinely prepared by bacterial expression thus permitting uniform labeling with the desired isotope (82). In addition, the heavy and light chains can be separately labeled, greatly improving spectral resolution. NMR analysis of MHC-II molecules has been hampered by the difficulty in producing these proteins by bacterial expression, although several groups have reported success in this area (55, 83).

The MD and structural features of peptide-free MHC molecules are of key importance for understanding the mechanism of peptide loading as peptide-free forms of MHC molecules are substrates for peptide loading and exchange by chaperones such as tapasin and TAPBPR for MHC-I and HLA-DM for MHC-II. However, it is challenging to produce peptide-free MHC-I molecules in amounts sufficient for detailed structural analyses and therefore information regarding their conformational dynamics has been largely obtained from MD stimulations [see, for example, Ref. (84, 85)]. An early biophysical and structural analysis of a peptide-free HLA-B*0702/$\beta_2$m heterodimer described an unstable, partially unfolded molecule in a molten globule state (86). More recently Kurimoto et al. (87) applied solution NMR techniques to peptide-free HLA-C*07:02/$\beta_2$m. NMR spectra obtained by selective labeling of methionine residues in the heavy chain revealed markedly attenuated intensities for residues in the peptide-binding domain suggestive of a partially folded molten globule form, whereas the α3 domain was properly conformed. These experiments highlight the role of the bound peptide in stabilizing MHC conformations for display at the cell surface to function as ligands for T cell and NK cell receptors.

## DYNAMICS OF pMHC-I

Although crystal structures of MHC-I molecules encompassing various allelomorphs and peptides show little gross variation, their analyses in solution by various biophysical methods and MD simulations indicate considerable differences in molecular flexibility at localized regions (7). Recent NMR analyses of pMHC-I complexes show heavy chain backbone as well as methyl side-chain dynamics revealing flexibility in exposed loops of the platform domain of the molecule (11).

The contribution of MHC-conformational dynamics to the relative dependence of MHC-I molecules on tapasin chaperone function for peptide loading has been addressed by comparative studies of HLA-B*44:02 and B*44:05 which differ only at position 116 (Asp for B*44:02 and Tyr for B*44:05) (88–90). These analyses suggested that HLA-B*44:05, which is tapasin-independent, preserves a peptide-free structure close to that of the peptide bound, even in the absence of tapasin.

A role for MHC-I conformational dynamics has been proposed to explain the differential disease susceptibility associated with two closely related HLA-B subtypes B*27:05 and B*27:09. Although the only amino acid sequence difference between the two subtypes is at position 116 in the floor of the peptide binding groove, which is Asp in B*27:05 and His in B*27:09, only B*27:05-expressing individuals are susceptible to ankylosing spondylitis (AS). Crystal structures of the two subtypes in complex with the same peptide are virtually identical. Using time-resolved fluorescence depolarization and MD simulations, Pohlmann et al. (91) showed that only peptide bound to the AS-associated subtype B*27:05 showed increased dynamics which is linked to the polymorphism at residue at 116. Thus, the increased dynamics is consistent with a molecule that has multiple conformational species that may aggregate either intra- or extracellularly contributing to various pathways to inflammatory disease.

Another point of difference between the B*27:05 and the B*27:09 subtypes is the dynamics at the $\beta_2$m-heavy chain interface revealed by NMR. Using isotopically labeled human $\beta_2$m, Beerbaum et al. (92, 93) compared the $\beta_2$m-heavy chain interface in the two closely related HLA-B subtypes, complexed with four different peptides, and found significant chemical shift differences in a $\beta_2$m loop that abuts the underside of the peptide binding groove and includes residues Asp53, Lys58, and Trp60. The most significant of these chemical shift differences is at Trp60 which shows subtype- and peptide-dependent structural variability. Conformational flexibility of $\beta_2$m at the interface with heavy chain, revealed by NMR, may thus influence peptide binding affinities and consequently MHC-I stability at the surface with important functional consequences for T cell and NK cell recognition. In addition, molecules that facilitate MHC-I peptide exchange and loading, such as tapasin and TAPBPR, may employ recognition of this $\beta_2$m-loop as a strategy to sense peptide occupancy, as discussed below.

Monoclonal antibodies that specifically recognize PR MHC-I molecules are valuable tools for identifying structural features

that correlate with the conformation of the PR state. Among the best studied examples is the 64-3-7 antibody which binds to immature, PR H2-$L^d$ but not to mature, PL H2-$L^d$ (94). The minimal epitope of 64-3-7 is a sequence of seven amino acids in the H2-$L^d$ α1 domain that adopts a $3_{10}$-helical conformation. Combining crystallographic, docking, and MD approaches, Mage et al. (95) showed that this $3_{10}$ helix moves in a hinge-like manner from an exposed and open position in the PR state to a closed position in PL molecules. The inward movement of the $3_{10}$ helix helps to form the A and B pockets that are crucial for stable peptide binding and subsequent release from tapasin in the PLC. It is noteworthy that the conformational dynamics of the $3_{10}$ helix occur at the opposite end of the groove from the site of tapasin binding—an illustration of the coordinated and dynamic changes that accompany peptide binding and chaperone release.

While computationally expensive, a wealth of information on the conformational flexibility of both peptide-bound and PR MHC molecules has been provided from all-atom MDs simulations in explicit solvent. In particular, in the absence of the peptide ligand, MHC-I molecules, such as HLA-A*02, HLA-B*44, HLA-B*27, H2-$D^d$, H2-$D^b$, and H2-$K^b$, exhibit increased mobility in the F-pocket region of the MHC, adjacent to the α2-1 helix (11, 84, 85, 96–99). Peptide-dependent dynamic coupling between the heavy chain groove and the α3/light chain interface has also been observed (100–102). Likewise, MD has uncovered similar conformational flexibility in the opposite end of peptide-deficient class II MHC molecules (HLA-DR1 and HLA-DR3) at the α51–59 and β58–69 regions (83, 103, 104). Finally, a putative role for N-linked glycosylation in modulating the local flexibility of the MHC groove has also been explored (105). Together, these studies show that polymorphisms within the MHC groove may dictate both ligand binding and overall allotype stability through alteration in dynamics, either in localized regions or globally. Taken together, these data indicate that modulation of MHC dynamics plays a defining role in peptide exchange, stability at the cell surface and co-receptor engagement where sparsely populated transient states may be involved (106).

## DYNAMICS OF PROTEINS OF THE ANTIGEN PRESENTATION PATHWAY

Newly synthesized MHC molecules are stabilized in a PR form in the PLC until loaded with high affinity peptide cargo. Following successful peptide loading, MHC-I molecules are released from the PLC and are transported through the Golgi to the cell surface. The PLC is a multimolecular, ER-membrane anchored assemblage consisting of the MHC-I/$\beta_2$m complex itself, the lectin calreticulin, the transporter TAP1/TAP2, the chaperone and peptide editor tapasin, and the disulfide isomerase ERp57 (see **Figure 1**). The molecular organization of this complex has been deduced from biochemical experiments (43, 44, 46), and X-ray structures of the individual proteins (107–111). Recently the structure of the PLC was visualized by cryo-EM (49) revealing an arrangement of the component proteins that is consistent with previous biochemically derived structural models (27, 104, 112), which indicate the association of a central TAP heterodimer with two peptide editing modules, each consisting of calreticulin, ERp57, tapasin and MHC-I. In addition, the cryo-EM images revealed intermediate states that lacked calreticulin and/or MHC-I, affirming the transient and dynamic nature of the molecular interactions within the PLC. A key component of the PLC is tapasin whose importance in selective loading of MHC-I with high affinity peptides is illustrated by the greatly reduced cell surface levels of MHC-I in tapasin-deficient cell lines (113, 114) and mutant mice (77). Binding to tapasin stabilizes MHC-I molecules that are peptide-free or suboptimally loaded (115) until an appropriate high affinity peptide is bound leading to tapasin dissociation from the complex.

Detailed mechanistic understanding of tapasin function in peptide loading is lacking because the structure of a PR MHC-I in complex with tapasin has proved elusive. Nevertheless, the structure of a tapasin–ERP57 complex combined with extensive mutagenesis data has revealed structural details of tapasin function (111). Tapasin is an L-shaped protein consisting of a membrane proximal Ig-domain and an N-terminal domain that is a fusion of a β-barrel and an Ig-domain. Differences in the orientation of the tapasin N- and C-terminal domains in the three copies in the asymmetric unit suggests interdomain flexibility is a structural feature of tapasin. The MHC-I interaction sites on tapasin, inferred from extensive mutagenesis data, reveal an evolutionarily conserved, extensive binding interface encompassing residues on both of the tapasin Ig domains. Combining MDs simulations of peptide-free MHC-I (84) and mutagenesis data identifying tapasin binding sites on MHC-I (116, 117), a structural model of the tapasin/MHC-I was proposed in which the primary focus of tapasin is the short helical segment of the MHC-I, α2-1, which is conformationally mobile and sensitive to groove occupancy (111).

More recently, mechanistic insights into MHC-I peptide loading and glimpses of the conformational dynamics involved have been obtained by crystal structures of the tapasin-like molecule, TAPBPR, in complex with PR forms of MHC-I (10, 12). Like tapasin, TAPBPR is widely expressed, interferon-γ inducible (118), and catalyzes the loading of high affinity peptides (119, 120). However, unlike tapasin, TAPBPR is not associated with the PLC (118) and TAPBPR-deficient cell lines display normal levels of MHC-I (119). Also, unlike tapasin, TAPBPR is not found only in the ER but also in the cis-Golgi (118). Although the role of TAPBPR in antigen presentation and its functional relationship to tapasin remain enigmatic, TAPBPR may function downstream of the PLC in conjunction with UDP-glucose:glycoprotein glucosyltransferase (UGGT1) (121), to provide additional peptide quality control.

The comparison of the three structures of H2-$D^d$: (1) occupied by a truncated, suboptimal peptide (pdb: 5WES); (2) peptide-free, stabilized by TAPBPR (pdb: 5WER); and (3) complexed with a high-affinity peptide (pdb: 3ECB) illustrates the conformational rearrangements that accompany the transition of MHC-I from a partially PL complex to a peptide-receptive and then to a PL state (10). In the TAPBPR-stabilized PR form, the MHC-I groove is widened in the region of the F pocket due to an ~3 Å displacement of the α2-1 helical segment. In addition,

β strands 5 and 8 that line the floor of the binding groove are displaced downward. The side chain of the conserved Tyr84 of MHC-I which in almost all pMHC-I structures coordinates both the C terminus of the peptide and Lys146 in the α2-1 helix is now flipped out of the groove to interact instead with Glu102 of TAPBPR (**Figures 2B,C**). Surprisingly, structural remodeling also occurs at the opposite end of the peptide binding groove as seen in the interaction between the side chains of Arg66 and Tyr159 which effectively close off this portion from peptide interaction. Extensive movements of the α3 domain and $\beta_2$m subunit also illustrate the differences between the PR and PL states. The 58–60 loop of $\beta_2$m which abuts the peptide binding platform from below and is conformationally dynamic and peptide-sensitive (92) forms key contacts to a hairpin loop of TAPBPR suggesting that peptide occupancy is sensed by TAPBPR through interaction with this $\beta_2$m loop. As modeled in the TAPBPR/H2-D$^b$ structure (12), peptide occupancy may also be sensed, and peptide loading facilitated, by a helix or loop of TAPBPR projecting into the groove near the F pocket. Finally, TAPBPR, like tapasin, has been suggested to stabilize the peptide-deficient MHC groove by dampening mobility of the α2-1 helix (122). Thus, as illustrated by both structures, coordinated and dynamic structural changes, stabilized transiently by TAPBPR interactions, occur during the critical step of MHC-I peptide loading.

A recent solution NMR study of the effects of the binding of TAPBPR to MHC-I (11) reveals stabilization of the dynamics of the empty MHC-I. On exposure to peptide, and with progressive peptide occupancy, the dynamics are further dampened, leading to an inverse relationship between MHC-I peptide occupancy and TAPBPR/MHC-I affinity. The NMR data reveal not only the interaction of conserved surfaces on the MHC-I heavy chain including the floor of the binding groove, the α2-1 helix, and the CD8 recognition loop of the α3 domain, but also effects on the $\alpha_1$ helix opposite the TAPBPR/tapasin binding site (and analogous to the HLA-DM binding site on MHC-II), all of which contribute to the widening of the binding groove in the chaperone-complexed but PR form of the MHC-I molecule. These results support a negative allostery release cycle as illustrated in **Figure 2**. In this mechanistic model, related in part to dynamics of the groove, the kinetic association rate of peptide binding to MHC-I is slow in the absence of a chaperone like tapasin or TAPBPR, and a peptide-receptive conformation is stabilized by the binding of the chaperone (**Figure 2A**). High affinity peptide binds rapidly to chaperone stabilized MHC-I, which ultimately releases the chaperone. A similar model is proposed for MHC-II binding to peptides, but in this case, the chaperone HLA-DM stabilizes the PR form of MHC-II by binding at the $3_{10}$ helix region (residues of the MHC-II β chain 80–93) (**Figures 2A,E,F**).

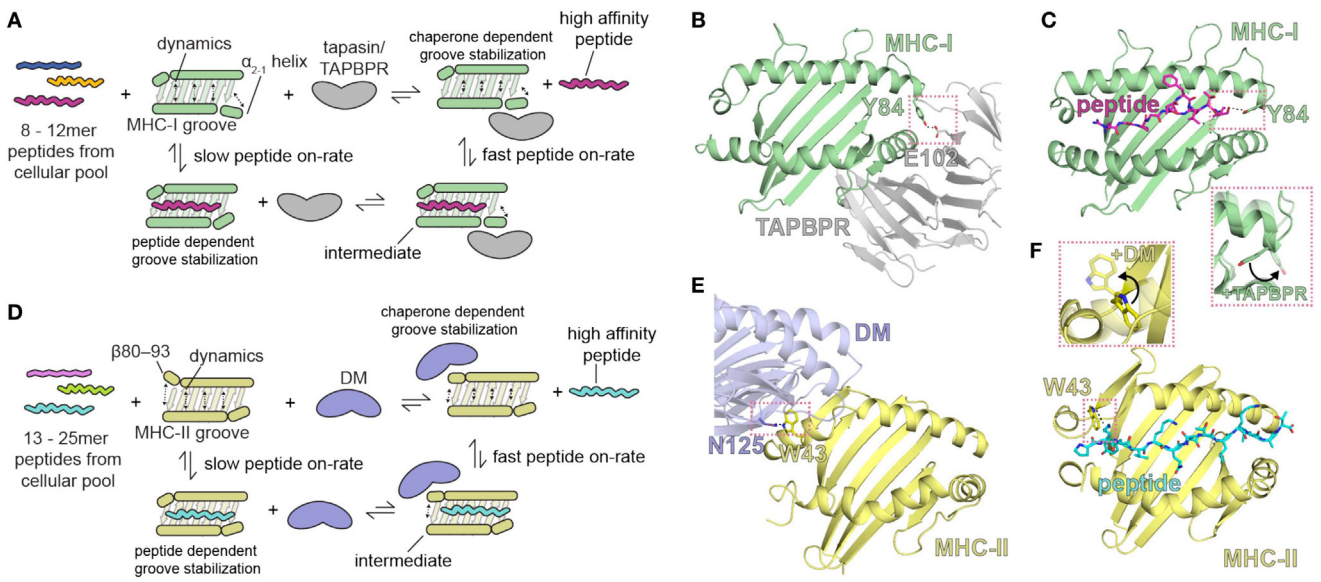


**FIGURE 2** | Summary of the role of major histocompatibility complex (MHC) groove structure and dynamics during peptide exchange and editing by the molecular chaperones tapasin or TAP-binding protein, related (TAPBPR). Schematic for class I **(A)** and class II **(D)** chaperone-assisted peptide exchange. Peptide exchange/editing occurs when peptides of 8–12 (class I) or 13–25 (class II) length are selected from the cellular pool in a manner highly dependent on the peptide sequence and the particular MHC allelomorph. This occurs either intrinsically (left) or is mediated by molecular chaperones (right) that increase the kinetic on-rate of peptides binding to the MHC groove through a stable intermediate complex. Conformational dynamics in localized regions of the MHC groove are stabilized by both the peptide and by molecular chaperones. The localized, dynamic regions including the α2-1 helix of MHC class I (MHC-I) and the β80-93 helix of MHC class II (MHC-II). **(B,E)** Structure of chaperone-bound MHC molecules with a focus on the MHC groove. Salmon dotted boxes highlight the conserved residues that are involved in chaperone-induced widening of the MHC groove (DM αN125 and MHC-II αW43; TAPBPR E102 and MHC-I Y84) relative to the peptide-bound state. **(C,F)** Structure of the peptide-bound (unchaperoned) MHC groove. Salmon dotted boxes highlight "flipping" of MHC-I Y84 and MHC-II W43 upon chaperone association, which in the absence of chaperones associate with the termini of the peptide. The chaperone-bound MHC states are shown as transparent in the inlays. PDB IDs are 5WER (H2-D$^d$/TAPBPR), 3ECB (P18/H2-D$^d$), 4FQX (HLA-DM/HLA-DR1), and 1DLH (Flu peptide/HLA-DR1).

A crucial step in the peptide-loading process is the trimming of peptides by the ERAP1 aminopeptidase (ERAAP in the mouse), the importance of which is underlined by the antigen presentation defect of ERAAP-deficient mice (47). Recent studies, exploiting both crystallography and MD simulations indicate the critical role of dynamic changes for the aminopeptidase activity of ERAP1 (123, 124).

## DYNAMIC ASPECTS OF T CELL RECOGNITION OF pMHC COMPLEXES

Once pMHC complexes have arrived at the surface of the APC, they are available for recognition by TCR or NKR. Most of our understanding of the molecular details by which TCR on the T cell or NKR on NK cells engage pMHC on APCs derives from crystallographic studies of TCR/pMHC (125) or NK/pMHC complexes (6). Pioneering efforts to explore dynamic aspects of the TCR/pMHC interaction used NMR chemical shift analysis to map the footprint of a pMHC-specific TCR onto its cognate MHC (126). These studies employed a truncated MHC-I molecule to identify chemical shift perturbations in solution that resulted from binding to a single chain TCR ligand (also ~25 kDa). The binding footprint obtained in solution in this manner was the same as that determined crystallographically for the same complex. In a complementary set of experiments, using NMR to examine residues of the same 2C TCR and of a labeled peptide in the pMHC complex, Hawse et al. (60) explored the dynamic changes that accompany the interaction of the pMHC with the TCR. They showed that structural fluctuations of the pMHC ligand matched similar fluctuations of the TCR, suggesting that TCR use these dynamic changes in solution to scan through different pMHC and to match those that have similar flexible modes.

## TCR CHANGES THAT ACCOMPANY pMHC INTERACTION AND COMMUNICATE SIGNAL TRANSDUCTION

In addition to studies of the pMHCpMHC interaction and TCR/pMHC interaction noted above, several groups have addressed the mechanism by which pMHC engagement by a TCR may contribute to signal transduction (**Figures 3A,B**). The TCR, in addition to consisting of αβ chains that recognize the pMHC, contains the ζζ homodimer, and the CD3γε and CD3δε heterodimers, as part of an eight-chain complex embedded in the T cell membrane. Cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) extend from ζ, γ, δ, and ε and, when phosphorylated by the lymphocyte-specific protein tyrosine kinase

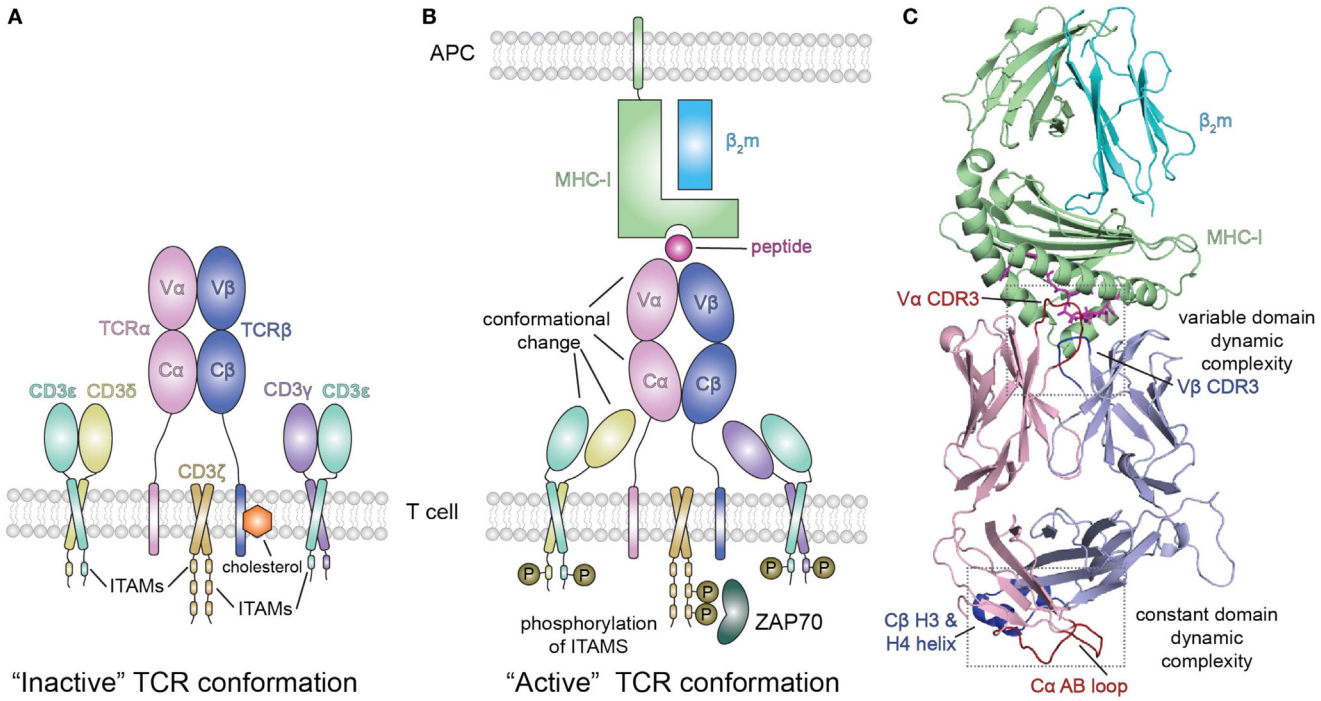


**FIGURE 3** | Peptide/MHC-I (pMHC-I) induced allosteric communication model for signaling of the TCR-CD3 complex. **(A)** In the absence of peptide–MHC-I ligand, cholesterol associates with the T cell receptor (TCR) β chain and maintains the TCR/CD3 complex in an "inactive" TCR conformation. The TCR αβ heterodimer is non-covalently associated with the CD3 γε and δε subunits as well as the ζζ homodimer. **(B)** Binding of pMHC-I molecules with the membrane distal variable domains (Vα, Vβ) of the αβ TCR receptor has been proposed to allosterically modulate the structure and dynamics of the membrane proximal constant domains (Cα, Cβ) which are sensed by the associated CD3 molecules resulting in an "active TCR." This pMHC-I/TCR mediated conformational change in CD3 allows for phosphorylation (P) of downstream immunoreceptor tyrosine-based activation motifs (ITAMs) that recruit proteins involved in signaling, such as ZAP70. Abbreviation: APC, antigen-presenting cell. **(C)** Structural view of the extracellular domain of the bound pMHC-I/TAP-binding protein, related complex (PDB ID 5IVX). Gray dotted boxes highlight regions of dynamic complexity that are proposed to be involved in the allosteric communication model.

(Lck), direct an activation cascade in the T cell. Aivazian and Stern (127) explored the lipid interaction of non-phosphorylated ζ chain and its mobilization from lipid vesicles when phosphorylated, suggesting that the availability of the ITAM for kinase activity was an early step in the extracellular binding of the eight chain TCR complex by a pMHC ligand. The structure of the cytoplasmic domain of the ζ chain has been explored in detergent micelles of LMPG and suggests that ITAM2 and ITAM3 interchange on the micro to millisecond timescale to regulate their accessibility for phosphorylation (128). Likewise, dynamic membrane associations that render the ITAM tyrosines inaccessible have been reported for the CD3ε cytoplasmic domain (129).

In efforts to explore the mechanism by which extracellular, cell-surface binding events convey conformational changes to cytoplasmic protein modules, three groups have explored changes in the CD3 (ζζ, γε, and δε) components of the TCR complex (13–15). He et al. (15) used an MHC-II-restricted αβ TCR isotopically labeled in the β chain to examine NMR chemical shift differences on exposure to γε and/or δε heterodimers. They observed small differences in a set of 9–11 solvent accessible Cβ residues consistent with a docking site requiring both γε and δε.

Using a different MHC-II-restricted TCR, others (14) labeled either the α or β chain and identified NMR spectroscopic changes in the constant regions consistent with δε docking on the Cα domain and γε on Cβ. These results were further supported by functional studies of mutagenized TCR in transfected T cells.

Extending this approach, Natarajan et al. (13) used a high affinity MHC-I restricted TCR to examine changes in the β chain TCR spectrum on pMHC binding. Remarkably, in addition to the dynamic changes of the interface residues of the TCR [the complementarity determining residues (CDRs)], these authors observed significant chemical shift changes in regions of the TCR remote from the pMHC interface, in particular near the Cβ H3 and H4 helices (**Figure 3C**). Confirmatory evidence was provided by site-directed mutagenesis and functional assays, consistent with an allosteric effect in the constant region resulting from pMHC-I engagement. The authors suggest that the allosteric transmission of conformational changes from the TCR CDRs in the variable domain to the Cβ distal sites occurs *via* the modulation of the variable/constant domain interface through the structural or dynamic rearrangement of the Vβ/Cβ linker regions.

## CONCLUSION

Biochemical evidence has long suggested that dynamic aspects of MHC molecules, the chaperones of the PLC, and the interactions with TCR might contribute to aspects of the functional molecular recognition steps throughout the entire MHC antigen presentation pathway. Only in the last few years have the combination of high resolution structural studies, computational MD, and multidimensional NMR been applied together to generate a mechanistic view of how conformational plasticity and MDs regulate multiple steps along the antigen processing and presentation pathway. It is now clear that molecular flexibility in peptide loading onto MHC, MHC/chaperone interaction, and pMHC interaction with TCR form a set of dynamic events contributing to their biological and potentially pathogenic role. A classical view of protein structure/function relationships ascribes function to the most stable (lowest energy) conformation. This understanding is being challenged by our appreciation that molecules that exhibit exceptional conformational diversity, known as intrinsically disordered proteins (IDPs) can represent a mixture of structured and unstructured regions or may even be entirely unstructured (130, 131). As a result, IDPs function by virtue of molecular associations that disregard traditional lock-and-key requirements and show flexibility in ligand binding. Studies of antigenic peptide dynamics, MHC-I and -II conformation changes, chaperone interactions, and pMHC-dependent TCR allostery now begin to reveal how dynamic or disordered regions of proteins contribute to their biological function. We expect that further studies of the molecular and cellular details of antigen processing, presentation and T cell signaling will shed light not only on this central aspect of the immune response, but also contribute to a more comprehensive understanding of how protein sequence, structure, and dynamics shape the biological function of macromolecules in general.

## A FINAL WORD

This review summarizes some of the enormous progress that the immunological community as a whole has made in addressing fundamental mechanisms of molecular recognition that initiate and propagate immune responses. Nevertheless, there remain complexities yet to be revealed as our understanding evolves from the specific to the general. Bill Paul had the unique ability to identify central problems whose solutions then would stimulate whole new areas of investigation. We trust that this review reflects in small part his continuing influence in encouraging us to study important questions and to seek definitive answers.

> "I believe that a leaf of grass is no less than the journey-work of the stars."*Leaves of Grass*, Walt Whitman

## AUTHOR CONTRIBUTIONS

KN, JJ, NAM, MGM, LFB, ACM, NGS, AB, and DHM conceived and wrote various parts of the review according to their expertise. KN, ACM, NGS, AB, and DHM edited and assembled the various contributions to the final text.

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