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Eric Vivier, *Aix-Marseille University*

Serge A. van de Pavert, *Aix-Marseille University*

[Max Cooper](#), *Emory University*

Gabrielle T. Belz, *Walter and Eliza Hall Institute of Medical Research*

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## The evolution of innate lymphoid cells

Eric Vivier<sup>1,2</sup>, Serge A van de Pavert<sup>1</sup>, Max D Cooper<sup>3</sup>, and Gabrielle T Belz<sup>4,5</sup>

<sup>1</sup>Centre d'Immunologie de Marseille-Luminy, Aix-Marseille University UM2, Inserm, U1104, CNRS UMR7280, Marseille, France

<sup>2</sup>Hôpital de la Conception, Assistance Publique–Hôpitaux de Marseille, Marseille, France

<sup>3</sup>Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA

<sup>4</sup>The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

<sup>5</sup>Department of Medical Biology, University of Melbourne, Parkville, Australia

### Abstract

Innate lymphoid cells (ILCs) are the most recently discovered group of immune cells. Understanding their biology poses many challenges. We discuss here the current knowledge on the appearance of ILC subsets during evolution and propose how the connection between ILCs and T cells contributes to the robustness of immunity and hence to the fitness of the hosts.

The recognition of lymphocytes was inarguably a major step in the understanding of immunity. This endeavor began in the first half of the 19th century when Gabriel Andral, a French professor of medicine, and William Addison, an English medical practitioner, independently reported the first description of leukocytes in 1843 (refs. 1,2). Then, Paul Ehrlich, pioneering a technique for staining and counting blood cells, identified myeloid cells and lymphoid cells as distinct white-blood-cell lineages<sup>3</sup>. It took almost another century to distinguish T cells and B cells as separate lymphocyte lineages in birds and mammals<sup>4–7</sup>. Natural killer (NK) cells were subsequently identified as non-T, non-B lymphocytes with the capacity for spontaneous or ‘natural’ cytotoxicity activity against tumor cells without the need for prior immunization<sup>8,9</sup>. The final discovery of the 20th century in the field of lymphoid cells was the identification of lymphoid-tissue-inducer (LTi) cells as a discrete subset of lymphoid cells that are essential for the development of peripheral lymph nodes and Peyer’s patches during embryonic life<sup>10</sup>. Then, in 2008 and 2009, 12 independent groups reported the identification in mammals of new types of non-T, non-B lymphocytes. These cells were called ‘innate lymphoid cells’ (ILCs), and this designation has been expanded to include the related subsets of ILC1 cells, ILC2 cells, ILC3 cells, NK cells and LTi cells<sup>11</sup> (Fig. 1).

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Correspondence should be addressed to E.V. (vivier@ciml.univ-mrs.fr) or G.T.B. (belz@wehi.edu.au).

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Since their identification, ILCs have been shown to contribute to defense against infection and to wound healing<sup>11–13</sup>. However, unlike adaptive immune cells, ILCs lack rearranged antigen-specific receptors, are rare in secondary lymphoid organs and exist mainly in non-lymphoid tissues as tissue-resident cells. The diversity of ILCs adds to the complexity of their analysis<sup>12,13</sup>, but striking similarities between ILC subsets and T cell subsets in the transcription factors that govern their differentiation and the cytokines they produce suggest that ILCs are the innate counterparts of T cells. Accordingly, it has been proposed that ILCs can be classified as cytotoxic ILCs, such as NK cells, and helper-like ILCs, such as the ILC1, ILC2 and ILC3 subsets. The coexistence of ILCs and T cells in mammals raises questions about when ILCs emerged relative to T cells during evolution and how the functions of ILCs and T cells are connected during immune responses.

Innate immunity is present in various forms in all plants and animals, and even single-celled organisms, bacteria, archaea and eukaryotes display protective immunological mechanisms. However, lymphocytes that circulate throughout the body via endothelial-cell-lined vessels to mediate adaptive immune responses have been found only in vertebrates<sup>14</sup>. Both jawless vertebrates and jawed vertebrates have prototypic T-like lymphocytes and B-like lymphocytes, which indicates that the basic genetic program for the development of T cells and B cells was already present in a common vertebrate ancestor ~500 million years ago<sup>15</sup>. However, for antigen recognition, lymphocytes in jawless vertebrates (lampreys and hagfish) use leucine-rich-repeat-based variable lymphocyte receptors as their antigen receptors instead of the immunoglobulin variable-diversity-joining-based receptors used by T lymphocytes and B lymphocytes in jawed vertebrates<sup>16</sup>.

ILCs have been characterized extensively in mice and humans, but so far there are no reports of ILC1s, ILC2s or ILC3s in other species. Before speculating on when the various types of ILCs began their co-evolution with T lymphocytes and B lymphocytes, we consider the information available at present and difficulties in the analysis of ILCs in evolutionarily diverse species.

## When did NK cells emerge?

NK cells represent the ILC subset for which there is the most extensive set of phylogenetic evidence. NK cells have been well defined in mammals on the basis of their potent cytotoxic function, target-cell specificities, activating and inhibitory receptors, cytokine requirements, cytokine production and transcriptional profiles<sup>17</sup>. Interestingly, the classical mammalian NK cell receptors are not conserved in other vertebrates, although some key effector molecules of cell cytotoxicity, such as Rab27a, perforin and granzymes, and transcription factors that regulate this cellular function, such as T-bet and Eomes, are more ancient (Fig. 2). Receptors of the NITR ('novel immune-type receptor') family, which are structurally related to mammalian receptors of the LILR ('leukocyte immunoglobulin-like receptor') and KIR ('killer-cell immunoglobulin-like receptor') families, have been characterized in bony fishes<sup>18</sup>. In addition, cytotoxic cells that are functionally similar to NK cells have been identified in several vertebrate species ranging from bony fishes, amphibians and reptiles to birds<sup>18</sup>. NK-cell-like cells, characterized by their morphology, lack of variable lymphocyte receptors (as antigen receptors) and transcriptional program, have also been identified in

lampreys (M. Hirano, J. Li and M.D.C., unpublished observations). Moreover, hemocytes with cytotoxic function and lymphocyte-like cell morphology have been described in tunicates<sup>19</sup>, the invertebrate chordates that are most closely related to vertebrates. Such findings, while inconclusive, are consistent with the idea that primordial NK-cell-like cells were present in a common ancestor of jawless and jawed vertebrates and possibly even in a common chordate ancestor of tunicates and vertebrates. Notably, the fragmentary evidence currently available indicates a gradual acquisition during vertebrate evolution of the distinguishing features of mammalian NK cells, including mechanisms of target recognition and cytotoxicity, cytokine profile and capacity for immunological memory.

## When did LTi cells emerge?

Although transcriptomic profiling and functional studies of humans and mice have suggested that LTi cells represent a subset of ILC3 cells<sup>20</sup>, published studies attest to their separate lineage status<sup>21</sup>. In lieu of formal characterization of ILCs in non-mammalian species, it is interesting to consider the evolutionary emergence of LTi cells from a functional viewpoint, since they are mandatory for the development of secondary lymphoid organs such as lymph nodes and Peyer's patches in mice<sup>20</sup>. Thus, it is tempting to associate the emergence of LTi cells with the emergence of these very organized secondary lymphoid structures. Of note, LTi cells are not necessary for formation of the spleen or of the tertiary lymphoid organs in mammals<sup>22</sup>. Although lymph nodes and Peyer's patches host the initial steps in the activation of T cells and B cells, their acquisition is a relative late event in the phylogeny of the vertebrate immune system. Indeed, these secondary lymphoid organs with separate B cell and T cell regions have been recognized only in some birds and mammals, which suggests they emerged ~100 million years ago<sup>23</sup>. Secondary lymphoid organization evolved earlier in jawed vertebrates, including the presence of splenic germinal centers in *Xenopus*, but these structures are different from mammalian secondary lymphoid structures. They resemble tertiary lymphoid organs, although they can contain germinal centers. Members of the tumor-necrosis-factor family, such as TNFSF11 (also known as RANKL or TRANCE) and lymphotoxins (LT $\alpha$  and LT $\beta$ ), are key LTi-cell factors for the organization of secondary lymphoid organs<sup>22</sup>. In contrast to TNFSF11, lymphotoxins appeared late in the evolution of jawed vertebrates (Fig. 2), coincident with the emergence of fully organized lymph nodes and intestinal Peyer's patches. Thus, it could be speculated that the acquisition of lymphotoxins was a limiting factor for the development of secondary lymphoid organs. In contrast, IL-22, a characteristic ILC3 cytokine, appeared in bony fishes<sup>24</sup>. ILC3 cells, therefore, could have evolved relatively early in the jawed vertebrates as mucosal innate cells that maintain epithelial integrity through the expression of IL-22. LTi cells with the ability to organize the development of peripheral lymph nodes and Peyer's patches through the production of lymphotoxins would then represent a later acquisition<sup>25</sup>. Consistent with this scenario, spleen- and gut-associated lymphoid tissues, which do not require lymphotoxin signaling for their development<sup>26</sup>, are universally present in extant jawed vertebrates.

## Dating ILCs via comparative transcriptomics

Despite the lack of a formal demonstration of ILCs in species other than mice and humans, a search for ILCs in non-mammalian species is possible through the use of transcriptomic, morphologic, phenotypic and functional analyses. One important determinant is *Id2*, a basic DNA-binding domain and a helix-loop-helix protein that promotes ILC development by antagonizing the E proteins that are essential transcription factors for T cells and B cells. In the mouse, all ILCs develop in an *Id2*-dependent way from a common lymphoid progenitor<sup>27</sup> (Fig. 1). Common lymphoid progenitors can differentiate via a common ILC precursor into three separate lineages: NK-cell precursor, LTi-cell precursor, and common helper innate lymphoid precursor. Cells of the last lineage express the transcription factor PLZF ('promyelocytic leukemia zinc finger') and lead to the ILC1, ILC2 and ILC3 subsets<sup>28,29</sup>.

ILC gene signatures can be identified in mice on the basis of 'preferential' gene expression in ILCs relative to gene expression in other hematopoietic populations<sup>30</sup>, and their conservation can be analyzed by the assignment of scores for the presence of orthologous genes in other species (Fig. 2). Beyond identifying conservation of the presence of *Id2* and *Plzf1*, comparative transcriptomics data have revealed remarkable conservation during vertebrate evolution of the presence of a large number of genes belonging to ILC transcriptomics signatures, such as *Nfil3* and *Gata3*, for the common ILC precursor; *Tnfrsf10*, for ILC1; *Ptprn13*, *Ar*, *Rxrg*, *Ccr8* and *Hs3st1*, for ILC2; *Cdh20*, *Kcnk1*, *Mapk10*, *Plekhhg1*, *Zmat4*, *Chad*, *Psd2*, *Cntn1*, *Dmrt1*, *Pram1* and *Tns4*, for ILC3; and *Rab27a*, for NK cell (Fig. 3). In the lamprey, lymphoid cells lacking variable lymphocyte receptors can be detected that express some of the markers found in mouse or human ILC subsets. Non-granular lymphoid cells lacking variable lymphocyte receptors can be identified that express lamprey orthologs of *Id2*, *Plzf* and *Rora* and express genes that encode transcription factors involved in mouse ILC2 development, whereas orthologs have not been found for genes encoding products required in mice for ILC1 and ILC3 cells, such as *Tbx21* (which encodes T-bet) and *Rorc* (which encodes ROR $\gamma$ t) (M. Hirano, J. Li and M.D.C., unpublished observations). Although these data should be completed by further analysis, they support the notion that whereas the ILC2 cells might have emerged in the most basal vertebrates, the full spectrum of ILCs seen in mammals might have evolved over time, leading to modifications in their gene-expression repertoire in various vertebrate species.

## Redundancy between innate and adaptive lymphocytes

If the emergence of ILCs began very early in vertebrate evolution and some types of ILCs preceded T cells and B cells, what are the advantages of adaptive lymphocytes relative to ILCs, and how are the functions of ILCs and adaptive lymphocytes connected? Innate lymphocytes and adaptive lymphocytes use distinct recognition strategies; i.e., germline-encoded receptors for ILCs versus rearranged antigen-specific receptors for T cells and B cells. The deadly clinical condition of severe combined immunodeficiency in patients who lack T cells and B cells demonstrates the non-redundant functions of adaptive lymphocytes in mammals<sup>31</sup>. Those data contrast with the lack of disease associated with ILC deficiency in humans (F. Vély, A. Fischer and E.V., unpublished observations). Thus, the continuous

evolution of adaptive lymphocytes has clearly added a selective advantage relative to that of ILCs in conferring protective immunity under current natural conditions.

Nonetheless, early studies of mice documented pivotal functions for ILCs in mucosal immunity, in particular in the context of gut infection with the Gram-negative bacterium *Citrobacter rodentium*<sup>32–34</sup>. However, the paucity of genes selectively expressed by ILCs or ILC subsets<sup>30</sup> and the consequent lack of mouse models for the selective targeting of ILC subsets have hindered delineation of the contributions of these cells to immune defense. In a unique mouse model with selective targeting of a natural-cytotoxic-receptor-bearing (NCR<sup>+</sup>) subset of ILC3, the NCR<sup>+</sup> ILC3 cells were found to be redundant with T cells for protection against intestinal infection with *C. rodentium*<sup>35,36</sup> (Fig. 3). Nevertheless, on their own, the NCR<sup>+</sup> ILC3 subset of cells was able to confer partial protection against bacterial infection. These observations suggest that the coevolution of ILCs and T cells involved the selection of redundant immunological mechanisms to ensure robust immunity. The selective pressure for redundancy might have led to features shared by subsets of ILCs and T cells, such as their localization in mucosal tissues and production of the same cytokines. Beyond ensuring robustness of the immune response, overlapping functions of ILCs and T cells might also limit the risk of immunopathology. Indeed, the ‘division of labor’ between cell subsets that share similar or complementary functions might limit the activation of individual lymphocyte subsets and hence reduce the likelihood of excessive subset activation that could lead to autoimmunity, as has been proposed<sup>37</sup>.

ILCs can also serve non-redundant functions that can be either deleterious or beneficial for the host. On the one hand, subsets of ILCs has been shown to be pivotal for the development of inflammatory diseases, such as ILC3 cells in the gut<sup>36,38,39</sup> and skin<sup>40</sup>, and ILC2 cells in the lungs<sup>41</sup>. On the other hand, the NCR<sup>+</sup> ILC3 cells are key to cecal homeostasis during infection with *C. rodentium*<sup>35</sup>, which shows that ILCs can serve non-redundant functions even in the presence of T cells. Of note, human ILCs are more abundant in fetal tissues and cord blood than in adults (F. Vély and E.V., unpublished observations). Such findings support the notion that ILCs might have especially important immunological roles when adaptive immunity is not yet optimal, such as when conventional T cells are not fully mature early in ontogeny or when these cells are otherwise compromised later in life. Along these lines, ILCs undergo substantial depletion in the circulation during acute viremic infection with human immunodeficiency virus type 1, and this deficiency might contribute to the immunological dysfunction observed in such patients with abnormal CD4<sup>+</sup> T cell counts<sup>42</sup>. The connection between ILCs and adaptive immunity is also demonstrated during the course of infection in mice, wherein ILCs initially respond rapidly to the challenge and are then ‘relayed’ by T cells for mounting protective immune responses<sup>43</sup>. Other modes of interplay between ILCs and T cells have also been reported, such as during hookworm infection, wherein ILC2s and T cells act together to ensure the maintenance of M2 macrophages for lung immunity<sup>44</sup>. Clearly, fine delineation of the complementarity and redundancies of ILC subsets and T cell subsets is one of the next frontiers in the field and will require the generation of animal models in which ILC subsets can be selectively targeted, as well as the genetic ‘experiments of nature’ in humans that should reveal the function of ILCs under natural conditions.



## Concluding remarks

In conclusion, the various types of ILCs identified in mammals are integral components of a multi-layered protective armory of immunocompetent lymphocytes that has emerged during vertebrate evolution. Comparative analyses further suggest that some of the ILC subsets, such as NK cells and ILC2 cells, were already present alongside the prototypic T lymphocytes and B lymphocytes in the most basal vertebrates. Still there are many gaps to be filled in the fragmentary evolutionary picture of lymphocyte development and diversification. The future identification and characterization of ILCs in a variety of vertebrate species other than mammals and possibly in the sister group of tunicates will be key to understanding how the remarkably diverse populations of lymphocytes evolved.

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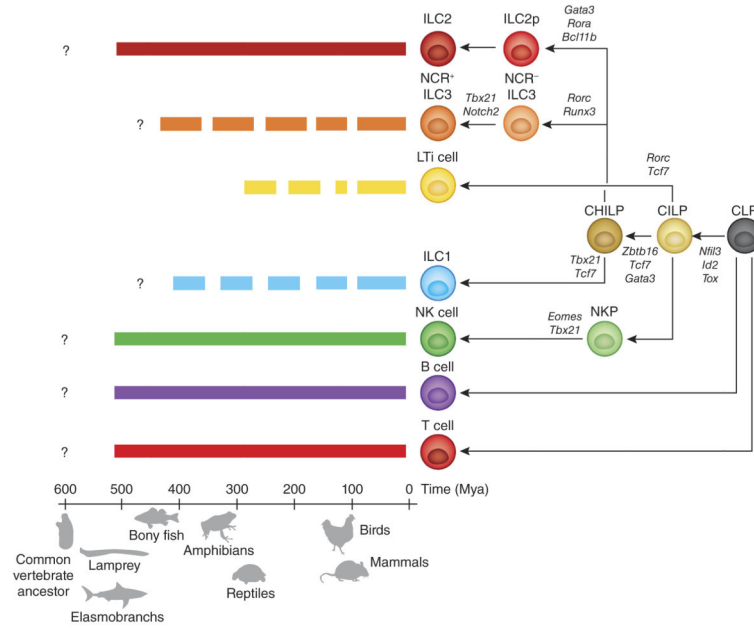
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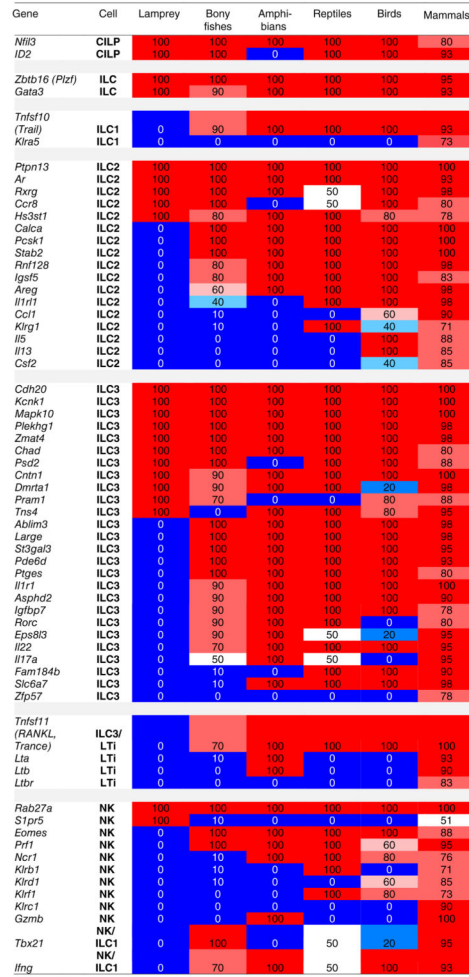
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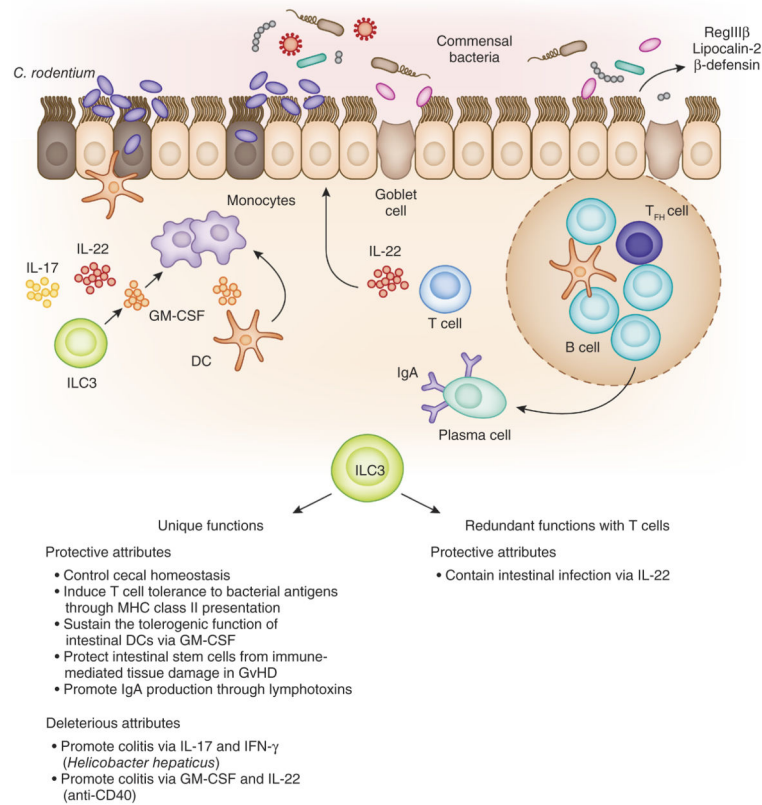
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**Figure 1.** Differentiation and evolution of ILCs. Model of lymphoid cell phylogeny (left), extending from the putative common vertebrate ancestor to birds and mammals (dashed lines and question marks indicate uncertainties), with the putative presence of certain cell types in some cases based on transcriptomics (for example, orthologs), and a model of ILC-differentiation pathways based mainly on transcriptional-program analysis in the mouse (right)<sup>29,41,42</sup>; line colores based on data in Figure 3. ILC2p, ILC2 precursor; CLP, common lymphoid progenitor; CILP, common ILC precursor; NKP, NK-cell precursor; CHILP, common helper innate lymphoid precursor; Mya, million years ago.



**Figure 2.** Phylogeny of ILC signature genes. Analysis of genes ‘preferentially’ expressed by the various types of ILCs or their progenitors in mice<sup>43</sup>, with an immunocyte-transcriptomic compendium built by merging of expression profiles available in the GEO genomics data repository<sup>44</sup>, plus the inclusion of genes encoding products shown to be involved in ILC differentiation (for example, *Id2*, *Nfil3*, *Gata3*), followed by a search for orthologous genes in lampreys, bony fishes (actinopterygii;  $n = 10$  species), amphibians (*Xenopus*;  $n = 1$  species), reptiles ( $n = 2$  species), birds ( $n = 5$  species) and mammals ( $n = 41$  species) by querying of the Biomart database from Ensembl (<http://www.biomart.org/>) and OrthoDB developed by the Zdobnov’s Computational Evolutionary Genomics group (<http://cegg.unige.ch/>). Numbers (and colors) in plot indicate frequency of a species in the family in which an orthologous gene has been identified.



**Figure 3.** Innate and adaptive lymphocytes exhibit both complementarity and redundancy in immunity. ILCs serve functions that are both distinct from and overlap those of adaptive immune cells; gut ILC3 cells serve as the example here. Intestinal T cells and ILC3 cells contribute to contain intestinal infections via their production of IL-22. The unique functions of ILC3 cells (bottom left) are based on published reports<sup>39,45–49</sup>. ILC3 cells can secrete the cytokines IL-17, IL-22, GM-CSF and lymphotoxins that contribute to protective immunity but also to inflammatory disorders via interaction with other cells, such as T cells<sup>46,49</sup>, dendritic cells<sup>47,49</sup> and neutrophils<sup>50</sup>. Precise delineation of the selective roles of NCR<sup>-</sup> ILC3 cells versus those of NCR<sup>+</sup> ILC3 cells has not yet been reported, with the exception of the control of cecal homeostasis upon infection with *C. rodentium*<sup>35</sup> and the promotion of inflammation in colitis induced by antibody to the costimulatory receptor CD40 (anti-CD40)<sup>36</sup>, which are properties of NCR<sup>+</sup> ILC3 cells. RegIIIβ, anti-microbial peptide; T<sub>FH</sub> cell, follicular helper T cell; DC, dendritic cell; IgA, immunoglobulin A; MHC, major histocompatibility complex; GvHD, graft-versus-host disease.