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# **ROR $\gamma$ t, a multitask nuclear receptor at mucosal surfaces**

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## **Summary**

ROR $\gamma$ t is a nuclear hormone receptor that has followed an exponential success carrier. Its modest origins as an orphan receptor cloned from human pancreas blossomed within 15 years into a critical regulator of anti-microbial immunity and a major target in the fight against inflammatory pathologies. Here, I review its role as a transcription factor required for the generation of type 3 lymphoid cells, which induce the development of lymphoid tissues, provide resistance of epithelial stem cells to injury, maintain homeostasis with the symbiotic microbiota, orchestrate defense against extracellular microbes and regulate allergic responses. ROR $\gamma$ t is also an intriguing molecule that is regulated by the circadian rhythm and includes cholesterol metabolites as ligands. ROR $\gamma$ t therefore links anti-microbial immunity with circadian rhythms and steroids, the logic of which remains to be understood.

## A brief history of ROR $\gamma$ t

Two independent lines of research lead to the discovery of ROR $\gamma$  and its shorter isoform ROR $\gamma$ t. In the first line of research, PCR primers were designed to amplify cDNAs coding for conserved regions of the DNA binding domain of nuclear hormone receptors. This strategy aimed for the cloning of presumably all nuclear receptors expressed in a given tissue or cell line. Using this approach, novel orphan receptors named retinoid Z receptor (RZR) $\alpha$  and RZR $\beta$  were then identified in umbilical vein endothelial cells<sup>1</sup> and rat brain<sup>2</sup>. In addition, using hybridization cloning with the cDNA of the retinoic acid receptor (RAR) $\alpha$ , retinoic acid related orphan receptor (ROR) $\alpha$  was identified in rat brain and shown to be identical to RZR $\alpha$ <sup>3</sup>. This led to the identification of the third member of the RZR/ROR family, ROR $\gamma$ , in a cDNA library from human pancreas, and later from mouse muscle, using the degenerate primer approach<sup>4,5</sup>. Interestingly, melatonin was reported to activate the transcription factor activity of ROR $\alpha$  and ROR $\beta$  in cultured cells<sup>6-8</sup>, suggesting early on a role for the ROR family of receptors in circadian rhythms.

In the second line of research, in a screen for proteins that confer resistance to TCR-mediated apoptosis, a shorter isoform of ROR $\gamma$  was identified by expression cloning of a thymocyte cDNA library in a T cell hybridoma<sup>9</sup>. While ROR $\gamma$  is expressed in a variety of organs, this new isoform was found to be primarily expressed in the thymus and was therefore named ROR $\gamma$ t. Mice that lacked the expression of both ROR $\gamma$  and ROR $\gamma$ t showed increased thymocyte apoptosis<sup>9-11</sup>. Such mice also had the remarkable phenotype of lacking lymph nodes and Peyer's patches. In knock-in mice in which the *Rorc*( $\gamma$ ) locus coded for GFP instead of ROR $\gamma$ t, but still expressed ROR $\gamma$ , it was shown that ROR $\gamma$ t is expressed by and is required for the development of fetal lymphoid tissue inducer (LTi) cells<sup>12</sup>, a then enigmatic cell type that colonizes developing lymphoid tissues in the fetus before any other hematopoietic cell type<sup>13-15</sup>.

It was nevertheless the association of ROR $\gamma$ t with Th17 cells that shot ROR $\gamma$ t to universal fame. In a landmark paper by Dan Cua and colleagues, it was demonstrated that IL-23, rather than IL-12, is the critical cytokine in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis<sup>16</sup>. In a second paper, Cua reported that IL-23 promotes the generation of Th17 cells, the effector cells that drive EAE<sup>17</sup>. On the basis of transcriptome profiles, Th17 cells were

found to express high levels of ROR $\gamma$ t as compared to Th1 cells, an observation that lead to the demonstration that Th17, like LTi cells, expressed ROR $\gamma$ t and required ROR $\gamma$ t for their generation<sup>18</sup>.

Two years later, the world of ROR $\gamma$ t<sup>+</sup> cells expanded again. A population of LTi-like cells was identified that express markers of NK cells, such as NKp46, as well as the signature cytokines IL-17 and IL-22, but do not cluster and induce the development of lymphoid tissues<sup>19-22</sup>. To keep control of the expanding universe of LTi-like cells, the term “innate lymphoid cells” (ILCs) was coined<sup>23, 24</sup>. It was progressively realized that the ILC universe includes ILC1s, ILC2s and (ROR $\gamma$ t<sup>+</sup>) ILC3s, which mirror Th1, Th2 and Th17 cells in the expression of signature transcription factors, surface markers and effector cytokines<sup>25</sup>.

A recent observation brings ROR $\gamma$ t back full circle to its original description as a cousin of the melatonin receptors ROR $\alpha$  and ROR $\beta$ <sup>6-8</sup>. ROR $\gamma$ t is a component of the transcriptional network of peripheral circadian clocks<sup>26, 27</sup>, which regulates the transcription factor Nfil3, which in turn represses the expression of ROR $\gamma$ t<sup>28</sup>. Deregulation of the circadian clock thus leads to a deregulation of Th17 cells and increased susceptibility to inflammatory pathology. Finally, and no less intriguing, cholesterol metabolites are natural ligands of ROR $\gamma$ t<sup>29, 30</sup>, possibly linking type 3 immunity to the endocrine system and metabolism, even though the biology of these ligands remains to be understood.

### **ROR $\gamma$ t and lymphoid tissue development**

LTi cells were first described as CD3<sup>-</sup>CD4<sup>+</sup> or CD3<sup>-</sup>IL-7R $\alpha$ <sup>+</sup> cells in the developing lymph nodes (LNs)<sup>13, 14</sup> and Peyer's patches (PPs)<sup>15</sup> (**Figure 1**). Mebius' and Nishikawa's labs characterized LTi cells and their interactions with stroma cells within the lymphoid tissue anlagen<sup>31, 32</sup>, but it is the discovery of ROR $\gamma$ t that allowed to demonstrate formally that LTi cells are required for the development of lymphoid tissues<sup>12</sup>. In the fetus, LTi cells are the only cells expressing ROR $\gamma$ t. In ROR $\gamma$ t-deficient mice, LTi cells are absent and LNs and PPs fail to develop. These cells express several members of the TNF superfamily, such as soluble lymphotoxin (LT)  $\alpha_3$  and its membrane-bound variant LT $\alpha_1\beta_2$ , TRANCE and TRANCE-L<sup>14, 31, 33</sup>. LT $\alpha_1\beta_2$  is essential for the development of lymphoid tissues<sup>34-36</sup>, as it engages LT $\beta$ R

on specialized stroma cells, which in turn induces the expression of adhesion molecules and chemokines that recruit lymphocytes and myeloid cells<sup>37</sup>.

After birth, LTi cells cluster in hundreds of so-called cryptopatches (CPs) located between crypts of the intestinal lamina propria<sup>38, 39</sup>. CPs collect B cells to develop into isolated lymphoid follicles (ILFs), which generate mostly T cell-independent IgA<sup>+</sup> B cells<sup>40-42</sup>. Surprisingly, the formation of ILFs from CPs requires bacterial microbiota<sup>41, 43</sup>. Proliferating bacteria release peptidoglycans from their cell wall, which is recognized by the innate receptor NOD-1 in epithelial cells<sup>44</sup>. This unleashes an activation cascade through the release of CCL-20, the activation of the CCR6<sup>+</sup> (the receptor for CCL-20) LTi cells and stroma cells in CPs and the recruitment of B cells<sup>43, 45</sup>. In ROR $\gamma$ t-deficient mice, ILFs-like structures still develop<sup>46</sup>. However, these structures are termed tertiary lymphoid tissues (tLTs), which are induced by chronic inflammation in most organs independently of LTi cells, through the expression of LT $\alpha$ <sub>1</sub> $\beta$ <sub>2</sub> by subsets of B cells, T cells or NK cells<sup>47</sup>.

Intriguingly, LTi cells are retained in mature (adult) LNs and Peyer's patches, and locate in the cortex between B cell follicles<sup>48</sup>. Their functions in this region remains enigmatic, mainly because it has been so far impossible to investigate the consequence of a absence of LTi cells in adult lymphoid tissues, as ROR $\gamma$ t-deficient mice lack both.

### **ILC3s**

A population of intestinal lymphoid cells expressing the NK marker NKp46 in mouse and NKp44 in human, were found to co-express ROR $\gamma$ t and IL-22<sup>19-22</sup>. These cells were originally named NK22 cells, but their requirement for ROR $\gamma$ t rather than Eomesin development, as well as their cytokine profile, makes them more similar to LTi cells than to NK cells. However, in contrast to LTi cells, NK22 do not cluster in CPs, and therefore, are not involved in the development of lymph nodes, Peyer's patches and ILFs<sup>49, 50</sup>. Rather, NK22 are viewed as more "regular" effector cells that patrol the tissue and produce effector cytokines where it matters in terms of defense and injury<sup>51</sup>.

Given their common dependence on ROR $\gamma$ t and their similar cytokine profiles, LTi cells and NK22 cells were grouped together as ILC3s, while lymphoid cells that produce the type 2 cytokines IL-4, IL-5 and IL-13 were grouped as ILC2s, and those

that produce IFN $\gamma$  as ILC1s<sup>23, 24</sup>. A difficulty arose when it was found that ILC3s can downregulate ROR $\gamma$ t and upregulate Tbet to produce IFN $\gamma$  in a context of intense inflammation<sup>52</sup>. These cells were termed ex-ILC3s. Another form of ILC3 plasticity was found *in vitro* using human ILC lines. When stimulated through TLR2, such lines expressed IL-5 and IL-13 in addition to IL-22<sup>53</sup>. It remains to be assessed to what extent such plasticity is operational *in vivo*, underlying for example the rapid response of ILCs to diverse types of tissue perturbations.

The prompt responsiveness of ILCs to infection and injury places them upstream in the immune response. ILC3s are activated by the inducer cytokines IL-1 $\beta$  and IL-23 produced by macrophages and DCs, typically in response to infection by extracellular bacteria and fungi. Activated ILC3s produce the effector cytokines IL-17, IL-22, GM-CSF, LT $\alpha_3$  and LT $\alpha_1\beta_2$ , which in turn induce the production of anti-microbial peptides (AMPs) by epithelial cells, of neutrophil-recruiting chemokines by stromal cells and epithelial cells<sup>25, 54</sup>, and the activation of B cells<sup>55, 56</sup>. This antigen-independent mode of activation of ILCs is similar to that of memory and “innate” T cells, at least in terms of kinetics<sup>57</sup>. It does not require selection and expansion of antigen-specific clones, but rather relies on the expansion of populations. It remains to be understood whether sub-subsets of ILCs exist and specifically expand in reaction to particular infections and injuries, thereby providing a form of immunological memory to challenges, as described for NK cells<sup>58</sup>.

ILC3s play a critical role early in the protection to enteropathogens, such as the proteobacteria *Citrobacter rodentium*, a homologue of the human *E. coli* intestinal pathogens<sup>19</sup>, to *Salmonella enterica*<sup>52</sup> and to rotaviruses<sup>59</sup>, through their production of IL-22. They also mediate containment of bacterial symbionts that live within Peyer’s patches<sup>60</sup>, and more generally protect from dextran sodium sulfate (DSS)-induced inflammation through the containment of symbiotic bacteria<sup>46, 61</sup>. The secretion by ILC3s of IL-22 also plays a role in the protection of epithelial cells from apoptosis induced by chemotherapy or irradiation in the intestine and the thymus<sup>62-64</sup>, as well as in the protection of hepatocytes from inflammation<sup>65</sup>. Intriguingly, ILC3s are relatively resistant to irradiation and chemotherapy, possibly as a consequence of their low turnover<sup>12, 49, 66</sup>.

On the other hand, ILC3s and IL-22 are involved in the progression of colon cancer<sup>67</sup>, presumably because they protect epithelial cells from apoptosis through the

activation of the transcription factor STAT-3<sup>62-64</sup>. In addition, ILC3s contribute to inflammatory pathology through their capacity to co-express IFN $\gamma$  during *Salmonella* infection in mouse<sup>52</sup> and in animals and patients suffering from inflammatory bowel disease (IBD)<sup>68, 69</sup>. The expression of IL-17 by ILC3s is involved in obesity-associated asthma induced by high fat diet<sup>70</sup>, possibly as a consequence of a loss of containment of the intestinal microbiota and an induction of ILC3s in adipose tissue by incoming bacteria and bacterial compounds<sup>71</sup>.

Another surprising feature of ILC3s is their expression of MHC class II<sup>12, 14</sup>, as well as of several components of the class II antigen-processing pathway. This feature allows the ILC3s to present antigens and repress the activity of specific T cells in the intestine during homeostasis<sup>72</sup>, and to induce T cells activation in the spleen<sup>73</sup>. The relative role of ILC3s, DCs and macrophages in the MHC class II-restricted activation and regulation of T cells<sup>74</sup>, at least in the intestine and the lymphoid tissues, remains to be measured.

So, ILC3s are pivotal to many processes in mucosal immunity, from the development of lymphoid tissues and the containment of the microbiota, the early immunity to pathogens and the protection of epithelial cells, to the exacerbation of inflammatory pathology and the progression of cancer. Even though ILC3s depend on ROR $\gamma$ t for their development, it remains to be determined whether ROR $\gamma$ t is also required for their maintenance, an important consideration when targeting ROR $\gamma$ t with agonists or antagonists to regulate type 3 immune responses<sup>75, 76</sup>. A recent report shows that a ROR $\gamma$ t antagonist leads to the loss of Th17 cells but not of ILC3s<sup>77</sup>.

### **Thymocytes and Th17 cells**

ROR $\gamma$ t is required for the survival of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes by regulating the level of the anti-apoptotic factor Bcl-x<sub>L</sub><sup>10</sup>. In the absence of ROR $\gamma$ t, immature thymocytes can spend less time at the CD4<sup>+</sup>CD8<sup>+</sup> stage and therefore, recombination of the genes coding for the TCR $\alpha$  chain is biased towards proximal V $\alpha$  to J $\alpha$  rearrangements<sup>78</sup>. The thymus of a ROR $\gamma$ t-deficient mouse contains half the number of cells found in the thymus of a wild-type animal<sup>10</sup>. In the periphery, control of anti-apoptotic genes by ROR $\gamma$ t has not been reported.

Expression of ROR $\gamma$ t by T cells remained confined to immature thymocytes, until Th17 cells develop that require ROR $\gamma$ t<sup>18</sup>. Naive CD4<sup>+</sup> T cells are induced into



the Th17 pathway by IL-1 $\beta$ <sup>79</sup>, IL-23<sup>17, 80</sup>, or the combination of IL-6 and TGF $\beta$ <sup>81-83</sup>. IL-23 and IL-6 induce the phosphorylation of STAT-3, which in turn induces the expression of ROR $\gamma$ <sup>t</sup><sup>84</sup>. The characterization of Th17 cells was initially reported in the context of EAE as the cells that are induced by IL-23 to express IL-17 and provoke autoimmunity<sup>17</sup>, as well as in arthritis<sup>85, 86</sup> and IBD<sup>87, 88</sup>. Therefore, ROR $\gamma$ <sup>t</sup> was first perceived as a public enemy that must be targeted to block the progression of autoimmune inflammation. Another line of research nevertheless showed the importance of Th17 cells in mucosal immunity to pathogens and their role in the containment of the microbiota through both the production of IL-17 and IL-22<sup>89, 90</sup>.

Caught in the middle of these confusing perceptions, IL-17 and IL-22 have been sometimes described as both pro- and anti-inflammatory, which only reflects the homeostatic or pathologic context in which these cytokines were studied. In contrast, ILC3s, also acting both during intestinal homeostasis and pathology, were first characterized in the context of intestinal homeostasis and thus as primarily “beneficial” cells<sup>39, 43</sup>. Nevertheless, the distinctive role of Th17 cells and ILC3s in homeostasis, defense and pathology has been difficult to pull apart, as models to ablate Th17 cells or ILC3s, individually, have to be improved<sup>19, 60</sup>. Based on their innate versus adaptive nature, early responses against pathogens have generally been “assigned” to ILC3s, while chronic responses, such as autoimmune inflammation, have been assigned to Th17 cells.

The mechanisms by which microbes induce Th17 cells, and more generally type 3 immune responses, remain a hard nut to crack. Whereas it is clear that IL-1 $\beta$  and IL-23 play a central role in the cascade of events that lead to the generation of Th17 cells<sup>17, 79, 80, 91</sup>, the signals that induce IL-23 are not known. It has been shown that ATP, produced by bacteria, activates a subset of intestinal lamina propria DCs through the P2X and P2Y receptors to produce IL-23<sup>92</sup>, but this mechanism remains to be validated in the context of bacteria that adhere to epithelial cells, such as segmented filamentous bacteria (SFB)<sup>93, 94</sup> and pathogenic strains of *E. coli*, which efficiently induce the generation of Th17 cells<sup>95</sup>. The pathway by which these adherent bacteria induce Th17 cells remains unknown.

### **Type 3 Tregs**

FoxP3 is the signature transcription factor for regulatory T (Treg) cells. Therefore, it came as a surprise that a significant proportion of intestinal FoxP3<sup>+</sup> T cells also express RORγt<sup>96</sup>. It was suggested that RORγt<sup>+</sup> FoxP3<sup>+</sup> cells are not Tregs, but rather precursor T cells that express both transcription factors until expression of one is promoted over the other to generate Tregs or Th17 cells<sup>83</sup>. This hypothesis was derived from the observation that 25% of intestinal Th17 cells had expressed FoxP3 at some stage of their development, as determined by genetic fate mapping of FoxP3<sup>+</sup> cells. It was also suggested that Tregs acquire characteristics of effector cells, induced by the inflammatory state of the tissue<sup>97</sup>. For example, colitis induces the expression of IL-23 by macrophages and DCs, which in turn induces the expression of RORγt in developing effector T cells as well as in Tregs. This leads to the expression of the chemokine receptor CCR6 on both mature Th17 and RORγt<sup>+</sup> Tregs<sup>96</sup>, and thus co-localization of both the effector and the regulator cells to augment the efficacy of immune regulation<sup>98</sup>. Furthermore, a small proportion of RORγt<sup>+</sup> Tregs was found to be “perverted” into genuine effector cells, induced by chronic inflammation into the expression of IL-17<sup>99</sup>.

Nevertheless, the majority of RORγt<sup>+</sup> Tregs produces IL-10 at levels that exceed the levels produced by other subsets of Tregs, and express high levels of other attributes of Tregs, such as ICOS, CTLA-4, CD39 and CD73<sup>100</sup>, and exert regulatory functions *in vitro* and *in vivo*<sup>96, 98</sup>. Moreover, IL-10-producing RORγt<sup>+</sup> Tregs dramatically expand during intestinal and lung inflammation, presumably to avoid exponential growth of the inflammation<sup>96</sup>. Nevertheless, it is possible that the phenomenon of Treg “perversion” expands during chronic long-term inflammation, conditions found in the intestine of IBD and colon cancer patients<sup>99</sup> - but rarely obtained in mouse models.

Recently, it was demonstrated that microbiota- and antigen-induced Tregs express RORγt, whereas microbiota-independent Tregs include a subset expressing Gata3<sup>100-103</sup>. The generation of RORγt<sup>+</sup> Tregs is dependent on antigen presentation by DCs and macrophages, as well as on the activation of STAT3<sup>100</sup>. Surprisingly, the “pro-Th17” cytokines IL-6 and IL-23, rather than IL-10, activate STAT3 for the generation of RORγt<sup>+</sup> Tregs. The eventual lineage choice to generate RORγt<sup>+</sup> Tregs instead of Th17 cells is dependent on the metabolism of vitamin A into retinoic acid

(RA), as an absence of vitamin A, or the inhibition of the RA receptor, tips the balance in favor of Th17 cells<sup>100</sup>. This suggests that the normal metabolism of vitamin A by DCs, stromal cells and neurons, rather than microbes, determines the balance between ROR $\gamma$ <sup>+</sup> Tregs and Th17, or in other words, between anti- and pro-inflammatory (type 3) responses. I therefore propose that RA is a “normo-signal”, required in many physiological processes, which is used by the immune system to monitor the health state of a tissue. In the context of tissue damage, upon infection or injury, the production of RA is decreased and the balance between ROR $\gamma$ <sup>+</sup> Tregs and Th17 cells is shifted in favor of Th17 cells. During homeostasis in the intestine, this balance is regulated to the level of type 3 immunity required to contain the symbiotic microbiota.

In one study, the absence of ROR $\gamma$ <sup>+</sup> Tregs lead to the increase in Th1 and Th17 cells, and the exacerbation of colitis<sup>101</sup>. However, in another study, ROR $\gamma$ <sup>+</sup> Tregs were not found to control Th1 and Th17 cells, but rather Th2 cells<sup>100</sup>. It was proposed that bacteria induce type 3 responses, mediated by Th17 cells and ROR $\gamma$ <sup>+</sup> Tregs, which collectively repress competing type 1 or type 2 responses, a phenomenon described by the equilibrium model of immunity<sup>104</sup>. As a consequence, in mice that lack ROR $\gamma$ <sup>+</sup> Tregs or Th17 cells, anti-helminth responses are increased and allergic inflammation is exacerbated. Thus microbiota regulates allergy through the induction of type 3 responses<sup>100</sup>. The exacerbated Th17 responses observed in the absence of ROR $\gamma$ <sup>+</sup> Tregs, reported in the first study<sup>101</sup>, may be the consequence of an intestinal microbiota that is more potently inducing type 3 responses than in the second study<sup>100</sup>.

The developmental origin of ROR $\gamma$ <sup>+</sup> Tregs remains debated and is difficult to address. Do they derive from Th17 cells, ROR $\gamma$ <sup>-</sup> FoxP3<sup>+</sup> Tregs, or naïve T cells? Genetic fate mapping, using *Foxp3* or *Rorc*( $\gamma$ ), cannot be conclusive, as FoxP3 is continuously expressed in the Treg lineage, whereas ROR $\gamma$  is already expressed by immature CD4<sup>+</sup>CD8<sup>+</sup> T cells in the thymus<sup>39</sup>. Resolution of the differentiation pathway of ROR $\gamma$ <sup>+</sup> Tregs requires the transfer of single T cells, or individual T cell barcoding.

### **Other cells expressing ROR $\gamma$**

ROR $\gamma$ t is expressed exclusively by lymphoid cells. Or so we thought. Populations of bone marrow and human blood neutrophils express ROR $\gamma$ t, as well as IL-17 upon stimulation with IL-6 and IL-23<sup>105</sup>. A similar subset of ROR $\gamma$ t<sup>+</sup> neutrophils was identified in mice that expresses IL-17 upon *Aspergillus fumigatus* infection of the lungs<sup>106</sup>. These observations are reminiscent of older data showing that LPS induces lung neutrophilia, the recruitment of which is induced by CXC chemokines produced by stromal and epithelial cells. These chemokines are in turn induced by IL-17 produced by neutrophils and T cells<sup>107</sup>.

### **ROR $\gamma$ t as a molecule**

The natural ligand for ROR $\gamma$ t has remained elusive for long (**Figure 2**). The related ROR $\alpha$  was co-crystallized with cholesterol and cholesterol sulfate<sup>108</sup>. Recently, it was found that cholesterol biosynthetic intermediates, such as oxysterols, are involved in the function of ROR $\gamma$ t during the development of lymph nodes and the differentiation of Th17 cells and IL-17<sup>+</sup> T $\gamma$  $\delta$ cells<sup>29, 109</sup>. These sterols are likely to be endogenous metabolites, rather than compounds derived from the microbiota, as lymph nodes and ILC3s develop in germ free animals. Interestingly, 7 $\alpha$ ,27-hydroxycholesterol, is both a ligand for ROR $\gamma$ t and EBI2, a G protein-coupled receptor involved in the positioning of activated B cells in lymph nodes. The functional link between ROR $\gamma$ t and EBI2 remains unexplored<sup>30</sup>.

Synthetic inverse agonists of ROR $\gamma$ t have been identified that derive from LXR agonists<sup>75</sup> or sterols such as digoxin<sup>76</sup> and ursolic acid<sup>110, 111</sup>. Such compounds trigger intense interests from the pharmaceutical industry as drugs to block type 3 responses during autoimmunity and IBD. Evidently, given the multiple cells and pathways that depend on ROR $\gamma$ t, including the containment of the intestinal microbiota and the regulation of competing immune responses such as allergic responses, such a strategy may cause important collateral damage, or be inefficient in the intestine<sup>46, 100</sup>. It is possible that for similar reasons, anti-IL-23 treatment is inefficient or even deleterious in the context of IBD, even though it is very efficient in the context of skin disease<sup>112</sup>. Interestingly, it appears that Th17 cells are more sensitive to transient ROR $\gamma$ t inhibition than ILC3s<sup>77</sup>, indicating that partial blockage of type 3 responses can be achieved with ROR $\gamma$ t inhibitors that may preserve the activity of ILC3s and some level of microbial containment.

Regulation of ROR $\gamma$ t also occurs at the protein-protein level. Itch is an E3 ubiquitin ligase that binds ROR $\gamma$ t and targets it for ubiquitination and degradation<sup>113</sup>. In the absence of Itch, mice develop IL-17-dependent intestinal inflammation and tumorigenesis. Nitric oxide (NO) also regulates the activity of ROR $\gamma$ t through nitration of tyrosine residues<sup>114</sup>. As a consequence, iNOS-deficient mice develop enhanced Th17 differentiation. At the transcription factor level, FoxP3 binds directly to ROR $\gamma$ t and appears to impose its anti-inflammatory program on ROR $\gamma$ t<sup>+</sup> Tregs over the pro-inflammatory program induced by ROR $\gamma$ t<sup>83, 96</sup>. Gata3, the signature transcription factor of type 2 responses, directly binds to and inhibits the *Rorc* promoter<sup>103</sup>. Finally, T-bet, the signature transcription factor of type 1 responses, prevents Runx1 to mediate transactivation of *Rorc*<sup>115</sup>.

An interesting twist in the regulation of ROR $\gamma$ t expression involves Nfil3. This basic leucine zipper transcription factor is required for the development of NK cells<sup>116</sup>, as well as more generally for the development of ILCs<sup>117-119</sup>. Nfil3 is negatively regulated by the clock protein Rev-Erba, and in turn, negatively regulates expression of ROR $\gamma$ t by directly binding to the *Rorc* promoter<sup>28</sup>. As a consequence, the expression of ROR $\gamma$ t, and the amplitude of type 3 responses, is regulated by the circadian clock, and disruption of the circadian rhythm leads to increased susceptibility to type 3 inflammatory pathology.

### **ROR $\gamma$ t and ROR $\gamma$**

ROR $\gamma$  directly regulates the expression of clock genes through binding to the promoter of *Cry1*, *Bmal1*, *Rev-Erba* and also *Nfil3*<sup>120</sup>. Thus, ROR $\gamma$  regulates clock genes, and one of those clock genes, *Nfil3*, regulates ROR $\gamma$ t<sup>28</sup>. However, ROR $\gamma$  and ROR $\gamma$ t are not usually co-expressed in the same type of cells. Whereas ROR $\gamma$ t is expressed in lymphoid cells (and neutrophils) exclusively, ROR $\gamma$  is not expressed in hematopoietic cells, but in many other types of parenchymal cells, such as hepatocytes and muscle cells<sup>9</sup>. Therefore, it is possible that ROR $\gamma$  and ROR $\gamma$ t have similar biology in different cells, both connected to circadian clocks and oxysterol ligands<sup>29, 109</sup>, but have different functions through the regulation of distinct set of tissue-specific genes.

Interestingly, the other members of the ROR family, ROR $\alpha$  and ROR $\beta$ , also play important roles in the circadian rhythms, as both ROR $\alpha$ -deficient mice and ROR $\beta$ -deficient mice show alterations in circadian oscillations<sup>121</sup>.

### **Concluding remarks**

ROR $\gamma$ t has turned out to be an extraordinary nuclear receptor and transcription factor, which controls type 3 immunity, a critical branch of immune responses that contains the symbiotic microbiota at mucosal surfaces and fights bacterial and fungal pathogens, but also leads to autoimmunity and cancer. The links between ROR $\gamma$ t, oxysterols and clock genes makes ROR $\gamma$ t a potential node connecting immunity with metabolism and circadian rhythms. The biology of ROR $\gamma$ t blossoms at an exciting time as immunology expands into a more transversal field of research connecting different physiological systems.

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## Figure legends

### Figure 1. The ROR $\gamma$ <sup>+</sup> cells.

ROR $\gamma$ <sup>+</sup> cells include type 3 innate lymphoid cells (ILC3s) and several subsets of T cells (Th17 cells, invariant NKT cells, T $\gamma$  $\delta$  cells). Not mentioned is a subset of IL-17-producing neutrophils that has been documented in mouse and man (see text). All ROR $\gamma$ <sup>+</sup> cells can express the effector cytokines IL-17 and IL-22 to varying degrees, except most ROR $\gamma$ <sup>+</sup> regulatory T cells (microbiota-induced or iTregs) that express IL-10. A subset of ILC3s, named lymphoid tissue inducer (LTi) cells, expresses soluble (LT $\alpha$ <sub>3</sub>) and membrane-bound (LT $\alpha$ <sub>1</sub> $\beta$ <sub>2</sub>) lymphotoxin, as well as TRANCE and TRANCE ligand, which are involved in the development of lymphoid tissues in the fetus and after birth in the intestinal lamina propria and the activation of B cells. IL-17, as well as LT $\alpha$ <sub>1</sub> $\beta$ <sub>2</sub>, induces the recruitment of neutrophils, IL-22 prevents apoptosis of epithelial stem cells, and both IL-17 and IL-22 induce the production of anti-microbial peptides (AMPs) by epithelial cells. ROR $\gamma$ <sup>+</sup> Tregs are induced by the symbiotic microbiota and regulate competing type 1 and type 2 responses.

### Figure 2. The ROR $\gamma$ t molecule.

The type 3 inducer cytokines IL-23 and IL-6 induce the phosphorylation of the transcription factor STAT3, which then activates *Rorc*( $\gamma$ ), the gene coding for ROR $\gamma$ t that is essential for the generation of ILC3s and ROR $\gamma$ <sup>+</sup> T cells. ROR $\gamma$ t then induces the expression of the type 3 effector cytokines IL-17 and IL-22. FoxP3, the signature transcription factor of Treg cells, binds ROR $\gamma$ t and generally imposes a regulatory phenotype to FoxP3<sup>+</sup> ROR $\gamma$ <sup>+</sup>(Treg) cells. Similarly, Gata3 and T-bet, the signature transcription factor of type 2 and type 1 lymphoid cells, respectively, block the expression of ROR $\gamma$ t. The expression of ROR $\gamma$ t is also under control of the circadian rhythm, as Nfil3, regulated by clock genes, represses *Rorc*( $\gamma$ ). ROR $\gamma$  is involved in the regulation of circadian clocks, but is not normally expressed by hematopoietic cells. Natural ligands of ROR $\gamma$ t have recently been identified as oxysterols, the biology of which remains to be fully understood.