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Role of neurotrophic factor receptors in Innate Lymphoid Cell immunity

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I. RESUMO

O intestino humano é considerado o maior compartimento do sistema imunitário, tendo constantemente de enfrentar e responder de forma eficiente a sinais e perigos externos, como antigénios e moléculas imunomodulatórias provenientes de várias fontes, incluindo o nosso próprio microbioma intestinal (1). Isto deve-se ao lúmen intestinal estar em contato direto com tudo aquilo que é ingerido, funcionando como uma porta seletiva entre o meio-ambiente e o corpo e até mesmo como um potencial nicho onde bactérias patogénicas se podem desenvolver e levar a situações de doença, podendo inclusive ser considerado uma parte exterior do nosso corpo. Adicionalmente, o sistema imunitário tem de integrar e modular os sinais provenientes do microbioma intestinal, que coevoluiu com os seus hospedeiros, estabelecendo uma relação simbiótica com eles de várias formas (2, 3), ajudando na manutenção da homeostasia, metabolizando compostos que de outra forma seriam indigeríveis, competindo e prevenindo que outras bactérias colonizem o trato intestinal e sendo até capaz de modular o próprio sistema imunitário (4). Em troca, nós oferecemos-lhes uma fonte estável e constante de nutrientes e um ambiente relativamente estável onde podem crescer e proliferar. (4). No entanto, em certas condições, quando o sistema imunitário não consegue regular e controlar devidamente as bactérias comensais, algumas delas podem tornar-se patogénicas (5). Ainda assim, é notável a forma como o sistema imunitário consegue lidar simultaneamente com as bactérias comensais e bactérias patogénicas, de forma a garantir a homeostasia intestinal em situações simultaneamente tão semelhantes e diversas. É fácil de imaginar que o que observamos hoje em dia foi o resultado de uma enorme pressão evolutiva para garantir o bom funcionamento do nosso sistema imunitário intestinal.

Entre as várias células que fazem parte do sistema imune inato e adaptativo e que têm um papel fundamental na regulação e manutenção da homeostasia, existe uma família emergente de células inatas de morfologia linfoide, as *Innate Lymphoid Cells* (ILCs). O seu papel em processos biológicos tem vindo a ser revelado ao longo destes últimos anos. Sabe-se atualmente que as ILCs tem um papel na iniciação, mediação e resolução de estados inflamatórios, integram sinais do microbioma, têm um papel na formação e reparação de órgãos linfoides, reconhecem e produzem citocinas imunomodulatórias e são inclusive capazes de modular a resposta do sistema imune adaptativo. As ILCs identificam-se pela ausência de marcadores clássicos de células B, T, mieloides ou granulócitos. No entanto, expressam alguns marcadores presentes noutros leucócitos, como no caso da cadeia gamma (γc, CD132), IL-7Rα (CD127), IL-2Rα (CD25), e Thy1 (CD90). Esta família de células foi recentemente dividida em 3 grupos, de acordo com a expressão de fatores de transcrição específicos e perfis de expressão de citocinas. Atualmente, considera-se existirem ILCs de tipo 1, 2 e 3 (6-14).

As ILCs de tipo 3 são um grupo relativamente heterogéneo de células, encontrando-se divididas em ILC3s e em células *Lymphoid Tissue Inducer* (células LTi). Estas células fazem parte do sistema imunitário das mucosas, estando presentes no intestino delgado e grosso e sendo produtoras de IL-22 (15). As ILC3s são definidas em ratinhos como sendo Lin^- $ROR\gamma t^+$ (e também $Thy1^+$ $IL\text{-}7R\alpha^{int}$ $C\text{-}Kit^{int}$ $CCR6^-$, com uma percentagem sendo $NKp46^+$), enquanto que as células LTi são caracterizadas como Lin^- $ROR\gamma t^+$ $NKp46^-$ (e também $Thy1^+$ $IL\text{-}7R\alpha^{hi}$ $C\text{-}Kit^{hi}$ $CXCR5^+$ $CCR6^+$), com uma percentagem sendo $CD4^+$. Este subtipo de células foi considerado como o grupo mais importante na produção de IL-22 numa situação de estado estacionário (16) e como tendo um papel extremamente importante na organogénese linfoide no feto (17-19), sendo ainda potentes produtores de IL-22 na fase adulta em ratinhos (20). No nosso laboratório, foi verificado que estas células expressam um recetor de fatores neurotróficos – RET (21).

Também observámos que a percentagem de ILC3s $IL\text{-}22^+$ se encontra reduzida em ratinhos com uma deleção condicional de RET em células $ROR\gamma t^+$ (*Rorc-Cre* $Ret^{flox/flox}$). Estes ratinhos foram denominados Ret^{Δ}. Em contraste, ratinhos com uma mutação genética em que existe um ganho de função de RET ($Ret^{MEN2B/MEN2B}$), em que o recetor se encontra ativo de forma constitutiva, apresentavam uma maior percentagem de ILC3s $IL\text{-}22^+$. Isto correlacionou-se com a reatividade epitelial destes ratinhos, sendo que a ausência de RET especificamente em ILC3s levava a que existisse uma menor expressão de genes associados à integridade epitelial. Pelo contrário, ativação constitutiva de RET levava a que existisse uma maior expressão de genes associados à reatividade epitelial. Isto fez-nos pensar que o RET tem um papel na produção de IL-22 em ILC3s, e que seria essa proteína a principal responsável pelas diferenças observadas, o que faz sentido se tivermos em conta o efeito que a IL-22 tem no epitélio. Esta proteína está descrita como tendo um papel antimicrobiano, regeneração de feridas e tecidos (22-24) e como sendo necessária para evitar a disseminação microbiana. (8, 25)

Com este trabalho, pretendemos portanto analisar se era esta alteração na produção de IL-22 que levava às diferenças observadas entre ratinhos mutantes e *wild type*, e não outro fator (igualmente dependente de RET) que não estava a ser tido em conta. Para conseguir isto, decidimos inicialmente induzir a produção de IL-22 em células ILC3 de ratinhos Ret^{Δ} de forma independente de RET, e verificar se seria possível "recuperar o fenótipo" observado em ratinhos *wild type*. Para tal, criámos um vírus capaz de infetar este tipo de células e induzir uma produção constitutiva de IL-22. Embora tenhamos conseguido infetar uma percentagem de ILC3s, tendo efetivamente desenvolvido, de acordo com o nosso conhecimento, o primeiro método para inserir genes neste tipo de células, o processo é relativamente stressante para

as mesmas e não nos foi possível recuperar um número suficiente de células para realizar um ensaio *in vivo*.

Paralelamente, desenhámos um método *in vitro* que nos permitiu estudar a interação de ILC3s com o epitélio intestinal. Foi-nos possível purificar estas células diretamente de ratinhos e realizar uma co-cultura com organoides intestinais (também conhecidos como “mini guts” - modelos *ex-vivo* do epitélio (26)), medindo a expressão de vários genes que são *upregulated* pela IL-22. Foi possível observar uma *upregulation* bastante evidente de alguns destes genes após uma co-cultura com ILC3s de ratinhos *wild type*, tanto constitutivamente como após uma estimulação com IL-23, o que nos permitiu validar o método de co-cultura e mostrar que as ILC3s tinham, de forma autónoma, a capacidade de estimular um aumento na reatividade epitelial. Esta estimulação encontrava-se bastante reduzida na presença de um anticorpo capaz de neutralizar o efeito de IL-22, o que indica que os efeitos observados dependiam diretamente da atividade de IL-22.

De seguida, estimulámos as células com ligandos de RET, e conseguimos observar um aumento na expressão destes mesmos genes, encontrando-se igualmente reduzida na presença do anticorpo anti-IL-22. Este efeito não se verificou em células cuja expressão de RET se encontrava afetada, o que fortaleceu a hipótese de que esta citocina é o fator que se encontra *downstream* de RET e que é responsável pelas alterações verificadas na reatividade epitelial de ratinhos com mutações neste recetor, indicando portanto que o RET tem um papel na produção de IL-22 em ILC3s e que essa produção está afetada quando a função de RET se encontra alterada, pelo que esta é a causa pelos diferentes fenótipos observados e descritos anteriormente.

Keywords: RET, Innate lymphoid cells, Interleukin-22, Epithelial Reactivity, Intestinal organoids

II. SUMMARY

The mammalian immune system has evolved to simultaneously allow for a peaceful cohabitation with the beneficial commensal bacteria, to provide defense against infectious agents and to initiate the repair and remodeling processes that restore and maintain tissue homeostasis.

Innate lymphoid cells (ILCs) are an emergent family of effector immune cells which display a lymphoid morphology, lack rearranged antigen receptors and are most abundant at mucosal surfaces. The combined expression of lineage-specific transcription factors along with specific cytokine profiles led to the formal classification of this family in three distinct ILC subsets: Group 1, 2 and 3 ILCs.

In the lab, it had previously been shown that RORγt^{+} ILC3s express the neurotrophic factor receptor RET. Furthermore, when the guts from *Ret*$^{GFP/+}$ mice (where GFP is knocked-in in the Ret locus) were analyzed by stereo microscopy, it was observed that GFP^{+} cells were located in aggregates, called Cryptopatches (CPs). It was also shown that RET is dispensable for the development of ILC3s (data not shown).

We have then shown that mice with a specific *Ret* deletion in RORγt-expressing cells (*Ret*$^{\Delta}$) show a decreased IL-22 production by ILC3s. Accordingly, mice with a gain-of-function mutant form of RET (*Ret*^{MEN2B}*,* where RET is constitutively active*)* have an increased percentage of IL-22^{+} ILC3s. Since IL-22 is known to induce the production of proteins and peptides (such as mucins and antimicrobial peptides) important for the maintenance of the epithelial barrier, we have also analyzed the gut of *Ret*$^{\Delta}$ and *Ret*^{MEN2B} mice and have found a strong reduction in the expression at the mRNA level of those genes in *Ret*$^{\Delta}$ mice and a marked increase in their expression in *Ret*^{MEN2B} mice. This data has shown that RET has a role in controlling innate IL-22 production and that the RET expression/activity modulates the epithelial reactivity.

We hypothesized that IL-22 was the link between RET and the changes in epithelial reactivity. In order to prove this, we have developed a virus capable of infecting these cells and inducing the constitutive expression of IL-22 in both *Ret*$^{\Delta}$ cells and Wild type (WT) cells, since we were interested in recovering the expression of IL-22 that is partially lost in cells that lack RET. In theory, this would allow us to recover the expression of genes that are upregulated by IL-22 either *in vivo* or *in vitro*. This would provide strong evidence that IL-22 is the molecular link downstream of RET, being directly responsible for controlling the epithelial reactivity. We were able to develop a method of ILC3s transduction (to the best of our knowledge, the first one), but it was not efficient enough for our planned *in vivo* trials.

In parallel, we have developed a novel co-culture system of intestinal organoids and ILC3s that allows us to study the interaction between them. The model was validated both by a rIL-22 stimulation and by a standard IL-23 stimulation of ILC3s, which showed that IL-22 is able to have a measurable effect on intestinal organoids and that stimulation of ILC3s was sufficient to increase some epithelial reactivity-related genes in this system in an IL-22-dependent manner.

We have also shown than RET stimulation is enough to induce the upregulation of those same genes. This upregulation was reduced in the presence of an IL-22-neutralizing antibody, which indicates that ILC3-autonomous RET signals are able to modulate the epithelial reactivity, maintaining the integrity of the epithelial barrier in an IL-22-dependent manner.

III. INTRODUCTION

1- Mucosal Immunity – General Aspects

The gastrointestinal tract is constantly exposed to various external challenges, microbes and antigens. As such, evolution has led to the development of a complex system, characterized by an interplay between immune cells and epithelial cells (and recently, the role of the microbiota is revealing itself to be extremely relevant as well) in maintaining the epithelial integrity and the intestinal homeostasis. This is essential to keep the pathogens out, the commensal bacteria controlled and for orchestrating inflammatory and anti-inflammatory responses, depending on the circumstances.

1.1 - Epithelial Barrier

The intestinal lumen is isolated by an epithelial layer (Fig. 1), which in turn is covered with a mucosal layer that is mainly produced and maintained by Goblet Cells. This layer is important in preventing the adhesion of harmful bacteria and in helping commensal bacteria thrive, contributing for the positive selection of beneficial bacteria in detriment of pathogenic strains. (27-30)

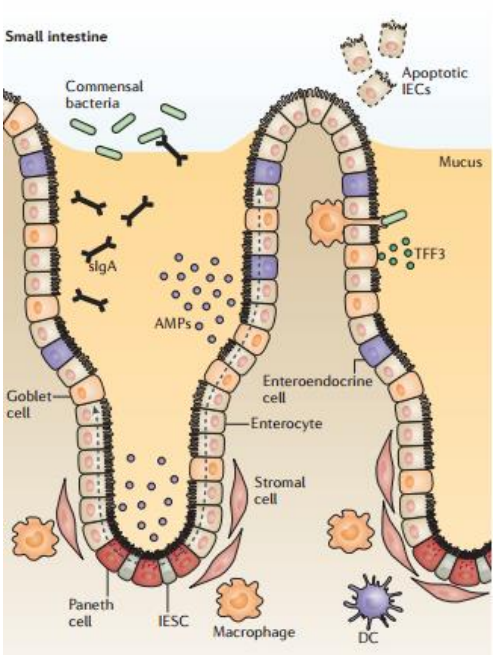

Figure 1: Schematic representation of the small intestine epithelium and the underlying Lamina Propria (31).

Aside from working as a physical barrier, epithelial cells also express various pattern-recognition receptors (32, 33). One could hypothesize that epithelial cells could downregulate and upregulate the various subtypes of Toll-like receptors (TLRs) expressed in response to different signals. If the host has some kind of mechanism capable of interpreting the various combinations of activation/non-activation of TLRs and making sense of them, this could represent a complex, intricate mechanism with the potential to be fine-tuned towards very specific situations - which is exactly what happens in the intestine. It could also mean the epithelium would have a way to control the host response against bacteria, giving these cells a certain plasticity and allowing them to have a different behavior depending on the circumstances, modulating their sensibility to certain stimuli and allowing for more specific and accurate responses.

While TLRs are mainly extracellular receptors, these cells also express nucleotide binding oligomerization domain-like receptors (NLRs) in their cytoplasm, which means there is also a way for these cells to sense when certain pathogens manage to enter the intracellular domain. Additionally, cells present in the epithelium are capable of both producing cytokines and responding to cytokines produced by other cells (22, 34, 35), some of which are responsible for inducing the production of mucins and antimicrobial peptides (36, 37).

It is possible to see that the epithelium is far from being a mere physical barrier capable of controlling the absorption of nutrients. In fact, it has a fundamental role in cooperating with immune cells to maintain the intestinal homeostasis.

1.2 - Lamina Propria

The *Lamina Propria* (LP) is the tissue immediately beneath the epithelial cell layer. Various immune cells locate at the LP, such as B cells, T cells, Innate Lymphoid Cells (ILCs) and Dendritic cells (DCs). The LP is an effector site, where the residing lymphocytes (the majority of which are T Cells) respond to stimuli (38), producing various cytokines such as IL-4, IFNγ, IL-17 and IL-22 (38, 39). It has been shown that T cells that are present in the *Lamina Propria* have markers of activation, such as $CD45RO^{+}$, $CD62^{low}$, $CD69^{high}$, CD25, $\alpha4\beta7^{+}$ and $CCR9^{+}$ (40), contributing to the notion that the LP is an effector site and other gut associated lymphoid tissues are activation sites.

Innate Lymphoid Cells are an important population in the LP. Among them, we have ILC1s (which might originate from ILC3 cells, being then considered ex-RORγt cells after the downregulation of this transcription factor), ILC2s, ILC3s and LTi cells, and the most well-known subtype of innate lymphoid cells, the NK cells.

Two important cytokines produced both by ILC3s in the LP are IL-17 and IL-22, which are able to have a direct effect in the epithelium, since epithelial cells express both IL-17R and IL-22R (41, 42).

Type 3 ILCs are further divided into 2 categories: ILC3s and LTi cells. ILC3s are defined in mice as Lin^- $ROR\gamma t^+$ $Thy1^+$ $IL\text{-}7R\alpha^{int}$ $C\text{-}Kit^{int}$ $CCR6^-$, with bimodal expression of Nkp46, while LTi cells are defined as Lin^- $ROR\gamma t^+$ $NKp46^-$ $CCR6^+$ (also $Thy1^+$ $IL\text{-}7R\alpha^{hi}$ $C\text{-}Kit^{hi}$ $CXCR5^+$), with a percentage of them being $CD4^+$. This subtype has been defined as the main IL-22 producers in the intestine in a steady state (16). These cells have even shown to be more important than their T cell counterparts in certain circumstances, like in the early stages of *Citrobacter Rodentium* infection(43, 44).

It is no coincidence that this tissue, which is extremely close to the epithelium, is very rich in various subtypes of lymphocytes: The *Lamina Propria* can be considered as a second line of defense, prepared in many ways to maintain and restore the integrity of the epithelium.

2 - Epithelial Barrier Cells

The intestinal epithelial barrier is far from being a uniform monolayer of cells. Instead, it is composed of various cell subtypes (Fig.2) which have different functions, all of them having an important role in the intestinal homeostasis.

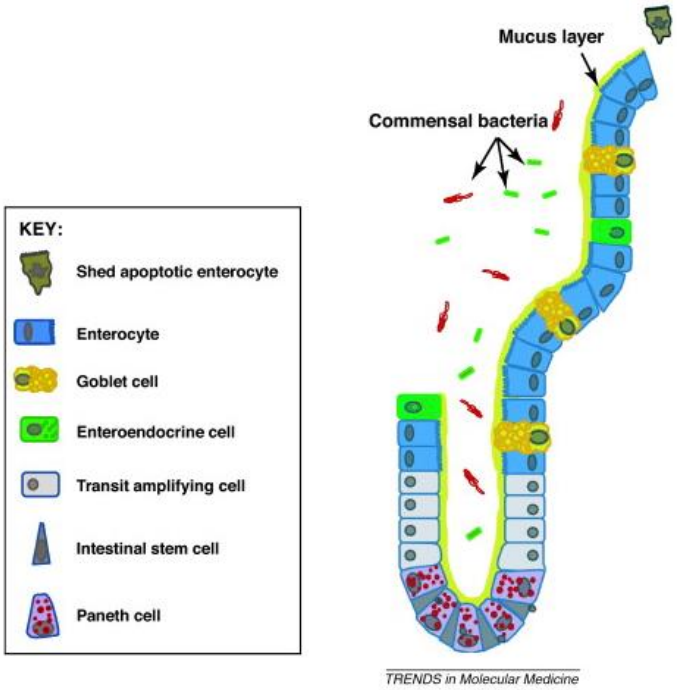

Figure 2: Schematic representation of a small section of the intestinal barrier, showing a crypt (bottom) and the tip of a villus (top), along with the various subtypes of cells and their localizations. The luminal side is represented by the location of the Mucus layer and the commensal bacteria (45).

The intestinal epithelium is one of the most rapidly self-renewing tissues in the human body, with a turnover time of about 3-5 days, being sustained by a population of intestinal stem cells (ISCs) that reside in the intestinal crypts (46). The crypt is a microenvironment which ensures the physical and biochemical signals essential for ISC maintenance, such as the Wnt, Notch and EGF pathways (47). This high rate of cellular differentiation is compensated by the equal high rate of cellular apoptosis at the top of the villus. The Wnt pathway plays an indispensable role in the maintenance of the normal intestinal architecture, and its inhibition results in the disruption of the cellular hierarchy and in loss of “stemness” by intestinal stem cells, characterized by a loss of their self-renewal properties (48, 49). In physiological conditions, each crypt contains about 5-20 stem cells, which have both the ability to self-renewal and to differentiate, giving origin to all the epithelial cells seen in Figure 2.

The most abundant cell type in the epithelium is the enterocyte, making for about 80% of the total cells of the epithelium. The enterocyte is a cell characterized by its polarization, held

together by tight junctions, capable of taking up antigens, expressing MHC I and MHC II (and presenting antigens to T cells) (50), and also appearing to express various TLRs (33, 51) and NOD1 (52).

Another major cell subtype are the Paneth cells, which are specialized in producing and secreting antimicrobial peptides (AMPs) that have a role in controlling the bacterial composition of the intestinal lumen, playing an important function in epithelial defense (53-56) and in maintaining the normal gut microbiota (55). Their frequency is higher near the intestinal crypts and, as a result, the zone closer to the crypt has a high concentration of AMPs. This helps preventing the bacterial invasion of these areas and maintaining the crypt microenvironment.

Goblet cells have a glandular morphology and exist along the total length of the intestine. Their main function is the production and maintenance of the mucus layer that coats the epithelium (57, 58), creating a so-called first line of defense against pathogens and a place that allows for commensal bacteria to thrive in a controlled manner (27, 29, 30). They do this by producing some specialized peptides, called Mucins, which are highly glycosylated molecules with gel-forming properties.

Making for about 1% of the total epithelial cells, enteroendocrine cells play a role in controlling the digestive process, by secreting peptide hormones that regulate the appetite and digestive responses (59, 60). There is evidence that they could also have important roles, directly or indirectly, in inflammatory processes, such as downregulating the appetite in situations of colitis (60, 61) or even by producing a peptide (GLP-2) that appears to promote epithelial repair and to shape the epithelial barrier, modulating the epithelium sensibility to TNFα (62, 63).

3 - Proteins and Peptides in the gut and their function

3.1 - Antimicrobial Peptides

AMPs are small peptides/proteins that have antimicrobial activity, working as part of the innate immune system. They rely on highly conserved structures and characteristics of pathogens, attacking them rapidly in a way that controls and/or neutralizes danger. This form of attack can either be done enzymatically, as in the case of Lysozyme, that is able to catalyze the breakdown of the peptidoglycan that constitutes the cell wall, an important structure that is needed for bacterial survival (64) and Phospholipase A2, an enzyme that acts directly on bacteria: it is able to penetrate the cell wall and catalyze the hydrolysis of the underlying membrane's phospholipids, strongly compromising its integrity and leading to death of the bacteria (65).

There are also other peptides that attack bacteria cell walls in a non-enzymatic way, such as defensins and c-type lectins (REG3 family). These proteins have a net positive charge at biological conditions, which allows them to interact easily with the bacterial cell walls, effectively exploiting its natural net negative charge (66, 67). While the mechanism by which C-type lectins work is still unknown, the mechanism for one of its members has been described recently. REG3A is able to penetrate the cell wall, associate with other REG3A molecules and form pores in the membrane (Fig. 4), eventually leading to osmotic lysis (68). It is possible that other members of the REG3 family share a similar mechanism.

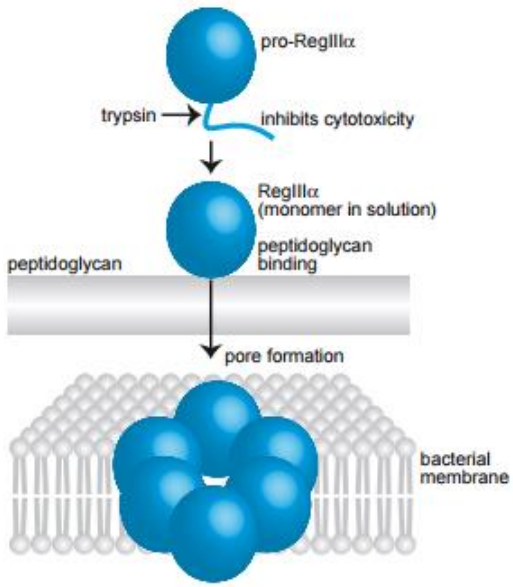

Figure 4: Bactericidal mechanism of REG3A: After being secreted in its inactive form, it is converted to the active form (as a monomer) by the action of trypsin. It is then able to cross the bacterial cell wall and associate with other REG3A molecules at the bacteria's cell membrane, forming a pore that causes osmotic stress and eventually death by lysis (68).

A similar mechanism appears to be valid for human α-defensin: it appears to dimerize, forming pores and disrupting the membrane.

There are also AMPs that disrupt the synthesis of the bacterial cell wall, like Human beta-defensin 3, that was shown to inhibit a critical step of the cell wall precursors in Staphylococci (69).

This vast array of proteins and different mechanisms of bacterial attack is probably necessary to minimize the chance of bacterial resistance to one particular peptide or mechanism and to widen as much as possible the spectrum of pathogenic bacterial strains that are susceptible to AMPs. In fact, the existence of such a diverse array probably only exists due to previous evolutionary pressure, caused by the development of resistance to a specific peptide, that pressured animals to develop another alternative to deal with the (now resistant) pathogens. This “evolutionary war cycle” probably had a strong influence in the current plethora of AMPs in complex organisms.

The focus on highly conserved structures (that usually are essential for the fitness of the organism, which is why they tend to be highly conserved in the first place) is also an excellent way to ensure that the pathogens can’t easily develop another method that renders the proteins/peptides useless.

3.2 - Mucins

The epithelial layer of the intestine is covered by a mucosal layer, made of proteins called Mucins that have a natural tendency to form a gel. These mucins are produced by Goblet Cells and are absolutely essential in maintaining this mucosal layer, necessary for the prevention of the adhesion of harmful bacteria and for helping commensal bacteria to thrive, contributing for the positive selection of beneficial bacteria in detriment of pathogenic strains (27, 29, 30). In addition promoting the formation of this physical barrier, mucin polymers (disulphide-linked) also help to lubricate, prevent dehydration of the epithelium surface and contain specific ligands to bind pathogens.

Mucins are divided in 2 categories: the gel-forming mucins, which are glycosylated in the Golgi and then enter the secretory pathway, forming large polymers that are able to trap water and give the mucus layer its consistency (70). The most important gel-forming mucin in the intestine, both in mice and in humans is MUC2. There are also transmembrane mucins, which have both a cytoplasmic domain and an extracellular domain. In the intestine MUC3, MUC12 and MUC17 appear to be the major components of the intestinal glycocalyx, and are probably involved in cellular protection (70).

4 - Interleukin-22

Interleukin-22 (IL-22) is a cytokine that was first described around the year 2000, having also been named IL-TIF (IL-10-related T cell-derived inducible factor), since it was found to share 25% homology with IL-10 (71), an anti-inflammatory cytokine (72). This cytokine was found to be produced by various immune cell subtypes and found to have antimicrobial, wound-healing and tissue repairing properties (22-24), and well as being necessary to prevent bacterial dissemination throughout the body (8, 25).

4.1 - Biological Function

IL-22 seems to act exclusively on non-hematopoietic cells. It was found to have an antiapoptotic effect (both on intestinal and on hepatic cells), being able to upregulate the expression of certain key antiapoptotic genes (*Bcl-2, Bcl-xL, Mcl-1*), and a proliferative effect, through the upregulation of mitogenic genes (*c-myc, cyclin D1, Rb2, CDK4*) in a pathway dependent of STAT3 in hepatic cells (73) and of genes such as *pla2g5, birc5, myc, smo,* and *mcl1* in the inflamed intestine (28). It was also able to promote wound healing in the skin (74) and help in the regeneration of the tissue in colitis models (75). Its role as a stem cell growth and protective factor (76, 77) could, at least partly, explain why it helps tissues (in the case of the intestinal epithelium) regenerate faster after they are damaged: it protects the stem cells and helps their proliferation.

IL-22 has a broad importance in various organs and tissues, such as in the liver, where it was shown to ameliorate liver damage in many pathological situations, such as Liver ischemia–reperfusion injury, Nonalcoholic fatty liver disease, Alcoholic liver disease and Acute liver injury (78). It also has a biological function in the lung, such as in the protection of chlamydia infection, in the inflammatory response against cigarette smoke and in the lung injury induced by the P. aeruginosa (79-81). As said before, in the skin it promotes wound healing (82) and is also implicated in psoriasis (83).

In the intestine, aside from the healing and proliferative properties, it is also necessary for the control of the microbiota, preventing its uncontrolled dissemination to peripheral organs and in keeping pathogens away, essentially in the earlier stages of the infection (23-25, 84, 85).

IL-22 achieves this by acting directly on the intestinal epithelium, signaling through the IL-22R present on epithelial cells (42). It stimulates the production of several peptides that are essential for the homeostasis (such as Mucins) and antimicrobial action (AMPs), as described previously. More precisely, IL-22 is needed and/or can induce the production of various proteins and peptides, such as the mucins MUC1, MUC3, MUC4 and MUC5b (86), the AMPs

REG3B, REG3G, S100A8, S100A9 and even several genes associated with IBDs, which hints towards the importance that IL-22 has in the pathogenesis of these diseases (87).

5 - Innate Lymphoid Cells

ILCs are the most recently discovered cell of the immune system and have been the focus of extensive investigation in the last decade. They are part of the innate immune system, being characterized by a lymphoid cell morphology and by the lack of any classical markers that are used to identify other defined lymphocytes, being commonly referred as “lineage negative” cells. They also lack a classical antigen receptor like those of B and T cells (BCR and TCR, respectively) that is able to undergo V(D)J recombination (since they lack the RAG gene), making these cells unresponsive to specific antigens and instead responding to less-specific signals (88, 89) (Fig. 5).

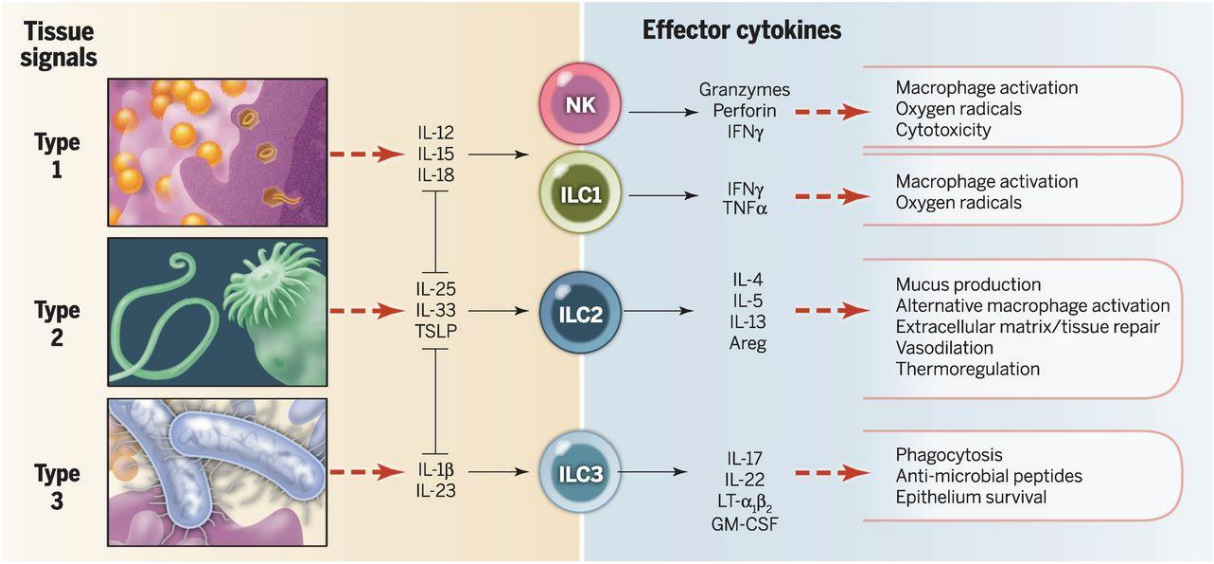

Figure 5: The type of alert cytokines associated with each pathogen and the way that innate lymphoid cells respond to them, with the corresponding global effect caused by that response (13).

ILCs express certain cytokine receptors and produce certain signature cytokines. This allows them to respond rapidly to “alert” cytokines and respond accordingly, making them a rapid, first line of defense that is able to respond before the adaptive immune system. That, along with certain surface markers and transcriptional factors is the currently accepted way to classify them. Another interesting characteristic is that they seem to have cytokine profiles that are similar to some helper T Cells (89).

5.1 - Group 1 ILCs:

Group 1 ILCs are characterized by the production of IFNγ and by the lack of expression of Th2 and Th17 signature cytokines (89, 90). The oldest known member of this category is the NK cell, which displays cytotoxic activity towards cells that express abnormal markers, indicative

of stress such as viral infection. They can also secrete TNFα and IFNγ (91), having a resemblance with cytotoxic CD8+ T cells.

The ILC1 is another (somewhat debated) member of this group. They are weakly cytotoxic (92) and are distinct from NK cells in the sense that they are not dependent on T-bet for differentiation and appear to be independent of Eomes, which is necessary for conventional NK cells development (93). However, the ILC1 is still a poorly defined and characterized type of cell and more studies will need to be done in order to further classify this population.

5.2 - Group 2 ILCs:

Group 2 ILCs were first identified around 2001, when a group reported a new population of "accessory cells" in $RAG^{-/-}$ mice that were able to respond to IL-25, producing IL-5 and IL-13. These cells were defined as "non-lymphoid accessory cells" by the authors, since they had no lineage markers, were $MHCII^{high}$, and $CD11^{null}$ (94). In 2010, some groups showed the existence of a novel type of cell, having been baptized with different names depending on the group that had described them, such as "Natural Helper" cells (10) or "Nuocytes" (95), having later been the subject of a revision, in which it was agreed to rename them to ILC2s, after it was concluded they were the same cell subtype, in an effort to give them a common name in order to facilitate further research in the field (89).

ILC2s respond to IL-25, IL-33 and TSLP by expanding and by secreting Type 2 cytokines, like IL-5 and IL-13 (96-98), making them critical early responders towards infections by extracellular parasites. This also makes them the "innate counterpart" of Th2 cells, which has served as the basis for their classification.

Interestingly, these cells also appear to control the response of Th2 cells, engaging in a crosstalk that is necessary both for a proper Th2 response for ILC2 expansion in vivo (99, 100).

5.3 - Group 3 ILCs:

Group 3 ILCs are defined by their expression of RORγt, needed for their development and for their function. Group 3 ILCs are somewhat more heterogeneous than ILC2s. This group contains both LTi cells, which are further divided into LTi0 and LTi4, depending on their lack or expression of CD4 (whose function is currently unknown), respectively, and ILC3s.

LTis appear during the embryonic stages and are required for the formation of Peyer's Patches and Lymph Nodes. They express CCR6, which is necessary for their clustering and

consequent formation of lymphoid tissues. They also express IL-17 and IL-22 during the embryonic stage, but the relevance of this expression is unknown, since mice that lack these cytokines are still able to develop lymphoid structures normally (101).

After birth, another subtype of Group 3 ILCs develops. These are defined as ILC3s and are an effector population that is essentially present on mucosal tissues, such as the *Lamina Propria* of the intestine. This population is again further divided in 2 other populations (The $Nkp46^+$ ILC3s and the $Nkp46^-$ ILC3s) depending on their expression (or lack thereof) of Nkp46, known as a NK cell activation marker, whose relevance is also currently unknown for ILC3s (102). Like the previous 2 subsets of ILCs, ILC3s also resemble a helper T cell, the Th17.

It was also shown that these cells can, depending on environmental cues, upregulate T-bet and become $Nkp46^+$ IFNγ-producing cells (102, 103).

5.3.1 - ILC3s in Intestinal Homeostasis

ILC3s are necessary for the maintenance of intestinal homeostasis and appear to be implicated in Inflammatory Bowel Diseases (IBDs). Both populations of ILC3s are known to be important producers of IL-22 and are key players during infection by *C. Rodentium* in mice (43, 44) and in DSS-induced colitis. They are activated by IL-23 both *in vivo* and *in vitro*, resulting in a strong production of IL-22. It is known that IL-23 is produced by DCs and macrophages in certain infections, like *C. Rodentium* (84, 104) which elucidates a way for these cells to respond during an infection.

As mentioned earlier, the immune system has a way to avoid responding to commensal bacteria as if they were pathogenic. These cells are no exception: it has been shown and proposed that IL-25 expression by epithelial cells downregulates IL-22 and IL-17 expression in ILC3s, in a DC-dependent manner (16). It was also shown that ILC3s express MHC II, and that ablation of this receptor results into abnormal responses of T cells towards commensal bacteria (9). This shows that ILC3s are not only important during immune responses, but are also important in steady state, being able to present peptides of commensal bacteria to T cells and regulating their response.

This allows us to get a glimpse at the network of cellular communication involved in the tolerance process that takes place in the intestine. It will certainly be quite remarkable to see this network further unravel as further research is published, allowing us to progressively understand how the intestinal immune system is able to differentiate between pathogenic agents and non-harmful, beneficial bacteria and/or antigens.

5.3.2 - T-Cell independent IL-22 production by ILC3s:

While T cells are a major source of IL-22 in several pathogenic states, this is usually a "late" response that takes time to reach full potential. Without an early, potent IL-22 production, infections become lethal and elimination of the pathogen is severely compromised or even impossible. This shows the importance of the existence of an IL-22 source that is able to act fast and efficiently, containing infections before the adaptive immune system has a chance to "kick-in" and deliver a potent, large scale response. This happens in several situations, such as *C. albicans* and *C. Rodentium* infection in the gut and *K. pneumonia* in the lung (44, 105, 106).

As said before, IL-22 is an important cytokine with effects throughout the body in epithelial tissues. Due to the nature of the work, I will only mention the IL-22 innate producers in the intestinal *Lamina Propria*, even though some of them have a role in other tissues and/or organs.

In the intestinal *Lamina Propria,* there is a specific ILC subtype that is able to produce IL-22 (Fig. 6). These are all part of the ILC3 family, with perhaps the exception of an ex-RORγt cell, which displays a phenotype similar to that of ILC1s, after the downregulation of RORγt and upregulation of T-bet and expression of IFNγ (102, 103, 107). All the other currently known subtypes of ILC3s have been described to produce IL-22 in one way or another.

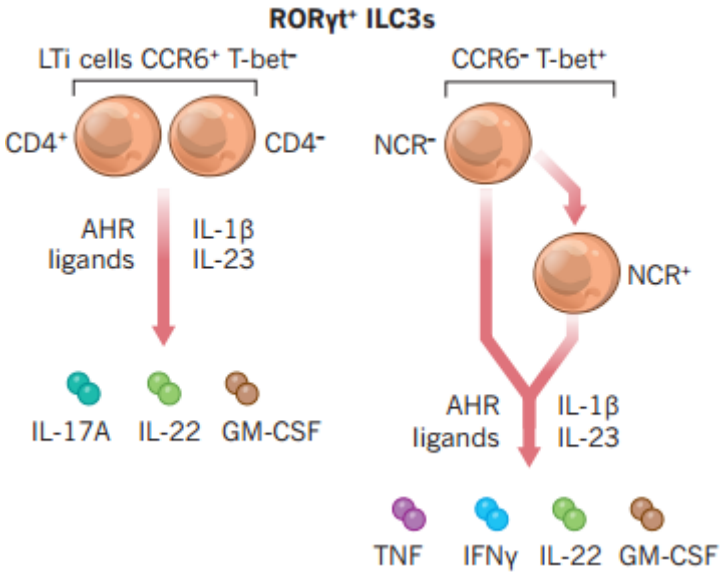

Figure 6: Adult mouse IL-22 producing ILC3s and the currently known subtypes that are part of this family (88).

Over the years ILC3s were defined and recognized by the expression (or lack thereof) of some markers whose purpose in ILC3s is not understood (CD4 and Nkp46), even though they are still regarded as members of this family. This led to a certain confusion and makes these cells

a likely target for a nomenclature review, as more is understood about the function of their surface markers.

$CD4^+$ LTi-like cells have been shown to be an important source of IL-22 in *C. Rodentium* infection, with their depletion leading to an impaired anti-microbial response (108). $CD4^-$ ILC3s cells have also been shown to be important IL-22 producers (20) in the murine gut. LTi cells have been described to produce IL-22 in the embryonic gut, even though the role of IL-22 at this stage is unknown, as these mice still seem to develop normally after IL-22 is ablated (101).

$Nkp46^+$ ILC3s also produce IL-22 and appear to be dispensable in certain conditions (103), in which ILC3-derived IL-22 production is essential.

All in all, the ILC3 family is still far from defined, and more research will need to be done in this area in order to further elucidate the subtypes of ILC3s, their function, the relationship between them and other ILC subtypes and the role of some proteins (such as CD4 and Nkp46, and possibly other still unknown ones) in ILC3s function and development.

5.3.3 – ILC3s in Human Inflammatory Bowel Diseases:

Human studies regarding intestinal ILCs are still very recent and scarce. One recent study has reported that in Crohn's disease (CD), there is an accumulation of a type of ILC that had a low production of IL-22 and higher production of IL-17, when compared to healthy controls. This phenomenon did not happen in Ulcerative Colitis (UC) patients (109). It is possibly that an ILC imbalance or dysregulation that favors the accumulation of IL-17-producing cells in detriment of IL-22-producing cells has a role in the etiology of the disease. The fact that it does not seem to happen in UC patients is very interesting, since it could provide clues to understand how both diseases work in more detail and to what is the precise role of the innate immune system in both diseases.

Another interesting study shows that in humans with Crohn's disease, $ROR\gamma t^+$ ILCs purified from inflamed zones have a decreased IL-22 production when compared to $ROR\gamma t^+$ ILCs purified from non-inflamed zones, and that their IL-22 production was increased when they were co-cultured with macrophages with LPS stimulation (110). This could indicate that a deficient interaction between the microbiota, macrophages and ILCs could be causing for a lower IL-22 production and consequent inflammation.

It was also shown that human ILC3s can differentiate to ILC1s and vice versa, with a specific ILC1 subtype ($IL7R\alpha^+$) being increased in Crohn's disease patients. This could also be relevant for the disease, and one could suggest that a bias towards the differentiation of ILC3s to ILC1s could play a role in the inflammatory process (11).

It is becoming increasingly likely that ILCs have a role in the etiology of inflammatory bowel diseases. Even though they add another layer of complexity in order to fully understand these diseases' mechanisms, it will be important to study their precise role and their biology in order to determine if they are candidates for novel therapeutics and what importance do they have in the development and/or management of these diseases.

6 - Physiological aspects of RET

The *Ret* (REarranged during Transfection) gene encodes for the RET protein, which is a transmembrane receptor tyrosine kinase (RTK) for the glial cell line-derived neurotrophic factor family of ligands (GFLs) (21, 111) (Fig. 7). RTKs are a large family of proteins that are involved in various signal transduction pathways that mediate cellular processes such as proliferation, migration, differentiation, survival and metabolism (112, 113).

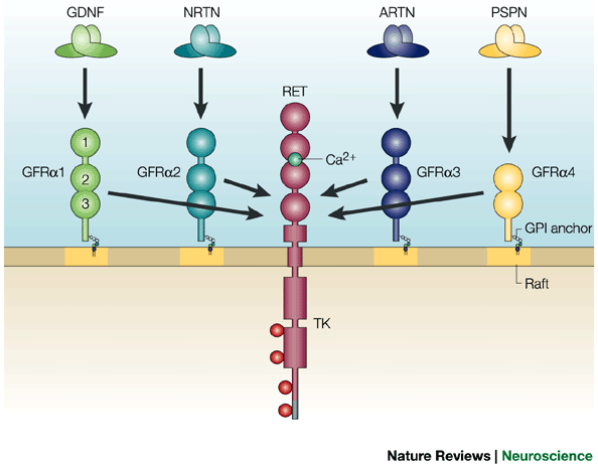

Figure 7: The transmembrane RET receptor and the 4 co-receptors, with their respective ligands (114).

6.1 - RET function:

The RET protein is a single-pass transmembrane receptor, that has an extracellular domain containing cadherin-like domains (transmembrane proteins that have a role in Ca^{2+}-dependent cell adhesion processes). So far, RET is known to be able to bind to 4 ligands: GDNF, Artemin (ARTN or ART), Neurturin (NRTN or NTN) and Persephin (PRSP or PSP). However, it cannot do this by itself. It also requires the presence of a co-receptor: GDNF-family receptor alpha (GFRα). Each GFL binds preferentially to a specific GFRα, as shown on Figure 7. This complex allows 2 RET molecules to form a homodimer, which is the process that promotes the autophosphorylation of the intracellular tyrosine residues.

However, this relationship doesn't appear to be the only way for GFLs to interact with RET, since there seems to be a certain promiscuity between ligands and co-receptors (115). GFRα can either act in *cis* when it is expressed by the cells expressing RET or it can act in *trans*, acting as a soluble receptor (116, 117).

The intracellular domain of RET is the kinase domain, where RET residues are able to undergo autophosphorylation upon RET activation (118, 119), a process that is necessary for the

interaction with several proteins that act as intermediaries in signaling cascades that are downstream of RET.

RET has also been shown to be expressed in hematopoietic cells, and was also shown to have a role in immune processes before. Mice with a deficient RET/GFRalpha3/ARTN pathway show impairment in the development of Peyer Patches (120). RET was also shown to be expressed in hematopoietic stem cells, regulating their survival and differentiation (121) and in Th2 cells, where it has a role in regulating their production of IL-10 (122). A broader study also analyzed the expression of RET in human T and B lymphocytes, monocytes and macrophages. While the study showed very high variation in RET expression in the same cell types between different individuals and its functional relevance was not analyzed, its expression seemed to positively correlate with the expression of IL-8, which is a cytokine expressed by monocytes and macrophages. The collection of these studies suggest that RET has a broad role in modulating immune cells and/or immune responses in ways that are far from fully understood. (123)

6.2 - RET-related diseases:

RET loss-of-function and gain-of-function mutants are implicated in some known diseases and conditions. MEN2 mutations, which result in a constitutively active form of RET, are divided in 2 categories: MEN2A and MEN2B. In MEN2A, the extracellular part of RET undergoes a mutation that allows 2 RET molecules to interact with each other, forming the homodimer necessary for signal transduction in a ligand-independent way. In MEN2B, the intracellular domain suffers a mutation which allows the kinase domain to phosphorylate the substrates without the need for homodimerization. This also results in RET activation independent of ligand. Both mutations lead to an abnormal activation of RET. Most patients (>90%) with these RET mutations display Medullary thyroid cancer and a smaller, but still very significative (about 50%) display pheochromocytoma (124).

In mutations that lead to a RET loss of function, it is possible to develop a condition called Hirschsprung disease (HRD), caused by a deficient development of an enteric nervous system due to a defect in Enteric Neural Crest Cell (ENC) migration during the development of the intestine. However, this disease does not appear to be exclusively caused by RET, and RET mutations are only present in 15-35% of HRD patients, with some other mutations associated with ENC being responsible for another 15-35% (in total, this makes for about 50% of HRD patients) (125, 126).

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